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PII: S0731-7085(18)31685-6
DOI: https://doi.org/10.1016/j.jpba.2018.09.048
Reference: PBA 12241

To appear in: *Journal of Pharmaceutical and Biomedical Analysis*

Received date: 19-7-2018
Revised date: 26-9-2018
Accepted date: 27-9-2018

Please cite this article as: Shirshahi V, Tabatabaei SN, Hatamie S, Saber R, Functionalized reduced graphene oxide as a lateral flow immunoassay label for one-step detection of Escherichia coli O157:H7, *Journal of Pharmaceutical and Biomedical Analysis* (2018), https://doi.org/10.1016/j.jpba.2018.09.048

Title: Functionalized reduced graphene oxide as a lateral flow immunoassay label for one-step detection of Escherichia coli O157:H7

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Highlights

- Use of graphene oxide and reduced graphene oxide as visual labels for lateral flow assay.
- Reduced graphene oxide shows higher visual signal than graphene oxide.
- It has been applied for one step detection of E. coli O157:H7 in food samples.

Abstract

In this study, graphene oxide (GO) and reduced graphene oxide (rGO) were used as visual labels in a lateral flow assay for detection of E. coli O157:H7. The color intensity was employed for the quantitative measurements of the target bacteria. Quantitative results showed that in comparison to GO, rGO can provide higher color intensity owing to enhanced light absorption following chemical reduction. Our results confirm that the visual limit of detection of the target bacteria by rGO is ~10^5 colony forming unit per milliliter (CFU/ml), which closely compares with current alternative techniques using gold nanoparticles. The performance and practicability of the rGO-based test strips for detection of the target bacteria in milk and drinking water were validated with conventional plating and colony

counting techniques. Results suggest that the proposed lateral flow assay is sensitive, specific, and affordable. It has also the potential to become a widely used detection technique for E. coli O157:H7 and a wide variety of other analytes.

Keywords: Point-of-care biosensor; Lateral flow assay; Reduced graphene oxide; Visual label

1. Introduction

In recent years, lateral flow immunoassay (LFA) as a point-of-care technology has been commercially successful and used for detection of a wide range of analytes and specimens in laboratory and filed [1]. Lateral flow test strips operate either in a sandwich or competitive format and they can detect analytes through an immunoassay reaction. In principle, this reaction occurs on a paper-based membrane and causes a color change observable by the naked eye [2]. To achieve efficiency in sensitivity, specificity, and reproducibility, choosing a suitable LFA label becomes critical. Conventionally, the colored reagents used in the majority of in-market LFA tests are metallic gold nanoparticles, polymeric latex particles, and dye colloidal particles [3]. However, these labels suffer from drawbacks such as

high cost of raw materials and low chemical stability. In the last few years, with the development of nanotechnologies, different nanoparticles have been used as labels for LFA test strips [4]. These may include magnetic nanoparticles [5], quantum dots [6], upconversion [7], lanthanide based fluorescent probes [8], and other multifunctional labels [9]. Although, they may provide higher sensitivity than the conventionally used labels currently available in the market, they face some drawbacks. For instance, magnetic nanoparticles require a rather expensive magnetic apparatus to separate and enrich the analyte to achieve higher sensitivity. In case of florescent labels, they require an ultra violet light source for excitation of the label and detection of the analyte. Moreover, fluorescent labels are not able to produce a visual signal. The need for additional apparatus and inability to be visually detectable has limited the simplicity and affordability of current LFA tests.

Carbon-based materials have been previously utilized as novel visual labels for LFA technology. In 1993, Van Amerongen et al. [10] illustrated the potential of colloidal carbon particles as a new label for LFA technology. Since then, carbon-based labels have been used in LFA tests for diagnosis [11], detection of microorganisms [12], and agrochemicals [13]. As a class of carbon-based nanomaterials, graphene derivatives including pristine graphene, graphene oxide

(GO), and reduced graphene oxide (rGO), are composed of single-atom thick sheets of carbon [14]. These two-dimensional nanomaterials have shown exceptional physicochemical properties with a wide variety of applications in many fields including biomedicine [15]. They are inexpensive, easy to prepare, highly stable, and have many oxygen containing chemical groups that give them high potency for labelling biological moieties such as proteins and nucleic acids. These properties promote the high potency of GO derivatives as new candidates for LFA labels [16]. In this investigation, rGO is used as a new label for LFA to detect E. coli O157:H7, a major cause of foodborne and waterborne infections [17].

rGO as an LFA label can provide major improvements over the currently used labels including gold nanoparticles. It has low preparation cost and reasonable stability in aqueous media. rGO does not need surfactants when it is partially reduced with a mild reductant like ascorbic acid [18]. It has also low density and provides good flow characteristics within LFA membranes and can be easily conjugated to biomolecules. In comparison to GO, rGO is black that can produce high color contrast on white LFA paper membranes. These properties would render disposable and inexpensive LFA test strips with high sensitivity, specificity, and reproducibility.

Table 1 compares advantages and disadvantages of common LFA labels with rGO. Currently, 40 nm gold nanoparticles are commercially used for many LFA tests. Their chemistry of synthesis and bioconjugation is well established and a number of products operate with these red color particles. However, gold salts as the raw material for the preparation of colloidal gold are expensive and a considerable amount of gold is thrown away by LFA strips when used in large scale.

Carbon-based labels are prepared by inexpensive raw materials such as graphite. They can provide a high visual intensity on LFA membranes due to the black on white signal produced on the membrane. The well-known carbon based label for LFA is carbon nanoparticles, which have substantial drawbacks preventing them from being widely used in the market. Some of these drawbacks include low stability and aggregation, low hydrophilicity, low density of functional groups, and low flow rates (Table 1).

This work illustrates the high potential of rGO as a novel label for LFA technology. rGO addresses advantages of carbon-based labels such as cheap raw materials, while overcoming their drawbacks. The preparation process is easy and well-known and a high amount of the nanomaterial can be synthesized each time. Carbon-based rGO is a low density material, thus it can migrate through LFA strips

easily and provide better flow rate. Therefore, in comparison with other carbon based labels, rGO can be successfully used as a label in LFA strips.

Tab. 1. Comparison of rGO label with some other LFA labels.

Label	Size (nm), flow rate	Advantage	Disadvantage	Analyte, Format	LOD	Ref.
Gold NPs	40, fast	Currently used, ease of synthesis, good biocompatibility, visually detectable and easy-to-read results	Low visual signal intensity and sensitivity, qualitative or semi-quantitative results, background interfering, need strip reader for quantitation, relatively high price	E. coli O157:H7, Sandwich	10^5 (CFU/ mL)	[19]
QDs	<10, fast	High sensitivity, strong photo-stability, wide absorption spectra and narrow emission bands, size-dependent optical properties	Need additional instruments, qualitative or semi-quantitative results, difficult to distinguish the result by the naked eye, difficulty of synthesis and conjugation	E. coli O157:H7, Sandwich	10^4 (CFU/ mL)	[6],[19]
Magnetic NPs	100 >, fast	Ease of functionalization, no degradation of signals over a long time	Need additional magnetic reader for quantitation	Various analytes	***	[2]

		period, low background noise				
GO-COOH	>1000, fast	Ease of synthesis, high density of functional groups, good biocompatibility, visually detectable and easy-to-read results, low price	Low visual signal intensity and sensitivity, qualitative or semi-quantitative results, background interfering, need strip reader for quantitation	Aflatoxin B1, Competitive	0.3 ng/mL	[16]
Carbon NPs	>150, slow	High visual contrast, good biocompatibility, visually detectable and easy-to-read results, low price	Low hydrophilicity, low density of functional groups, aggregation, low flow rate, qualitative or semi-quantitative results, background interfering, need strip reader for quantitation	phytoregulator forchlorfenuron (CPPU), Competitive	89 ng/L	[13], [20]
rGO	>1000, fast	High visual intensity, ease of synthesis, good biocompatibility, visually detectable and easy-to-read results, low price	Low hydrophilicity, low density of functional groups, qualitative or semi-quantitative results, background interfering, need strip reader for quantitation	E. coli O157:H7, Sandwich	10^5-10^6 (CFU/mL)	this work
NPs: nanoparticles QDs: quantum dots GO: graphene oxide rGO: reduced graphene oxide						

2. Material and methods

2.1. Chemical and materials

L-ascorbic acid and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Steinheim, Germany). Graphite powder (mesh ≤200 µm), potassium permanganate, ammonia solution, sulfuric acid, hydrogen peroxide, and sodium borate were purchased from Merck (Darmstadt, Germany). The chemicals were used without any additional purification unless noted. Cellulose fiber sample pads were purchased from Millipore (Burlington, Massachusetts, USA) and nitrocellulose membrane were purchased from Sartorius (Goettingen, Germany). E .coli monoclonal antibody (mAb), E. coli polyclonal antibody (pAb), and rabbit anti-mouse IgG secondary antibody (anti IgG) were purchased from Abnova (Taipei, Taiwan). E. coli O157: H7 and E. coli O20 were provided by Veterinary College of the University of Tehran (Tehran, Iran). E. coli ATCC 25922, Bacillus subtilis ATCC 19659, Staphylococcus epidermidis ATCC 12228, Pseudomonas aeruginosa ATCC 27853, and Salmonella typhimurium ATCC 14028 were purchased from Darvash (Tehran, Iran).

2.2. Preparation of GO and rGO

GO was prepared from expandable graphite powder as the initial material through a modified Hummers' method [21]. Generally, 1 g of graphite powder was ground with 50 g NaCl until a gray powder formed. Water was then added to the powder and the resulting suspension was filtered to remove NaCl. The prepared powder was dissolved in 23 ml of 98% H_2SO_4 for 8 h. Then, 3 g of $KMnO_4$ was gradually added to the solution. This process is extremely exothermic and it is important to keep the temperature under 20 °C to avoid an explosion. After stirring for 30 min at 40 °C and then 45 min at 70 °C, 46 mL water was added and stirred continuously for another 30 min at 98-105 °C. The reaction was terminated by adding 140 mL deionized water and 10 mL of 30% H_2O_2. The resulting mixture was washed once with 5% HCl, and then three times with deionized water. GO was reduced at 95 °C for 30 min by adding a 10 µL ascorbic acid and a 400 µL of ammonia. Finally, rGO was washed three times with deionized water.

2.3. Conjugation of GO and rGO to monoclonal antibody

A physical absorption approach was applied for conjugation of rGO to mAb [22], and the resulting conjugate was named rGO-Ab. Initially, 0.5 ml of rGO (1 mg/ml) suspended in borate buffer (2.5 mM, pH=8.8) was prepared and sonicated. Various amounts of mAb diluted in borate buffer (5 mM, pH=8.8) was added to the

suspension and the resulting mixture was gently shaken for 5 minutes. To this mixture, 250 µL BSA solution (10% in borate buffer, 5 mM, pH=8.8) was added to block unspecific bindings and the resulting mixture was sonicated shortly. Subsequently, the mixture was centrifuged at 15000 g (~13000 rpm) for 10 min to remove excess protein. The sediment was dissolved in borate buffer (5 mM, pH=8.8) having 2% BSA and stored at 4 °C. The above procedure was used for the conjugation of GO to mAb (GO-Ab) as well. Conjugation was confirmed by flowing the conjugates through lateral flow strips that had been coated with pAb and anti IgG as test and control zones. The accumulation of brown and black colors at the location of the control zone confirms functionalization of GO and rGO with mAb.

2.4. Preparation of LFA test strips and coating of reagents

In order to prepare LFA test strips, nitrocellulose paper (length 2 cm) was pasted to a PVC adhesive backing card and a sample cellulose fiber pad (length 1.5 cm) was pasted at the initial part of membranes. An absorption cellulose fiber pad (length 1.5 cm) was also pasted at the upper part of membranes with 2 mm overlap with the nitrocellulose membrane. Then, the prepared sheets were manually cut into separate strips (length 5 cm, width ~0.4 cm) by a paper cutter. 1 µL of pAb (1 mg/ml) per strip was manually dropped on nitrocellulose membrane as the test

zone. Similarly, 1 µL of anti IgG (1 mg/ml) was dropped on the membrane with a distance of ~0.5 cm above test zone as control zone (as circular dots). Finally, strips were incubated at 37 °C for 1 h. The prepared strips were stored at RT in a dry condition.

2.5. LFA procedure and optimization

Various concentrations of E. coli O157:H7 were prepared in running buffer (100 mM Borate buffer, 2% BSA). Then, 5 µL of the prepared rGO-based conjugates were mixed with 95 µL E. coli O157:H7 of each concentration in an ELISA well and gently shaken. Then the sample pad of the strips were placed vertically on the ELISA well until the mixture was absorbed by the membrane via capillary forces. Running the fluids through each strip (running time) lasted 20 minutes.

For all LFA experiments, the black color of the test zones resulting from rGO-based conjugate was determined visually as follows: the dot that appeared in the test zone was considered as a positive or semi-positive result based on the color intensity. If no dot was seen at the test zone, the result was considered as negative. A dot should always appear above the test zone to determine validation of the assay. The assay procedure for the GO-based conjugate was performed in a similar way.

For real sample analysis, samples of low fat milk and drinking water were confirmed to be free of bacteria by plate culturing. To minimize matrix effects, 1 ml of each real sample was diluted with 9 ml running buffer, from which samples with different concentrations of E. coli O157:H7 (0, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/ml) were prepared. The LFA procedure for real samples were performed similar to the procedure for standard bacteria solutions.

2.6. Quantitative procedure of rGO-based LFA

Test strips were imaged by a digital camera and transferred to a computer and then converted to 16 bit images by Image/Type/16-bit command of Image J software. To remove background and highlight the grayscale images, Process/Subtract background and then Image/Adjust/Threshold commands were used. In order to set a measurement for a zone, Analyze/Measure command was used. Intensity of test zones (IT) and control zones (IC) were measured for each strip. The standard curve was established by plotting the ratio of IT to IC (IT/IC) against the logarithm of the standard solutions of the E. coli O157:H7 concentration. The standard solutions were prepared by spiked E.coliO157H7 solutions (0, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/ml) in running buffer. The quantitative

analysis of real samples was calculated based on a linear equation of the E. coli O157:H7 standard curve.

2.7. Bacteria cell culture and counting

All bacteria strains were cultured in a MacConkey agar medium for 24 h at 37°C. Plate counting was used to determine total viable cells of the bacteria strains.

2.8. Characterization

Ultraviolet-visible (UV-Vis) spectra of GO, rGO, conjugated GO, and conjugated rGO were measured by a dual-beam UV/Vis spectrophotometer (Laxco Alpha-1900, China) in a quartz container from 200 nm to 1100 nm. Transmission electron microscopy (TEM) images were taken by an electron microscope (LEO 906, Oberkochen, Germany) operating at 80 keV. X-ray diffraction (XRD) of sample powders of GO and rGO for amounts of 2Θ from 5° to 80° in order to determine interlayer spacing was performed using a X'Pert MPD diffractometer (Philips, Amsterdam, Netherlands) at a voltage of 40 kV and a current of 40 mA with a cobalt anode and step size of 0.02. For Raman spectroscopy GO and rGO powders were placed in a Si/SiO_2 substrate. Raman measurements of GO and rGO was obtained at room temperature by a Takram P50C0R10 (Teksan, Tehran, Iran) with an emission laser of 532 nm emission wavelength and a power of 0.5 to 70 mW.

3. Results and discussion

The aim of this work is to evaluate the potential of rGO as a new type of visual label for LFA technology.

3.1. Preparation of GO and rGO

Single layer GO was chosen as the initial material. Low thickness of GO can provide better aqueous stability for the material, particularly when it is reduced or is exposed to ionic solutions during the conjugation process. Moreover, this is a prerequisite for having a fast flow of GO-based probes through the nitrocellulose membrane. GO was prepared by a modified Hummer's method as described earlier. TEM characterization confirmed that the lateral dimension of the GO, rGO and conjugated rGO sheets were around 1 µm (Fig. 1a,b and c). Form the TEM images, it is also conceivable that the graphene based layers are highly transparent indicating that the sheets are single layered.

Aqueous solution of concentrated GO is brown, but when the material is reduced, its color is changed to black. The black color of rGO can produce a higher contrast on the white nitrocellulose membrane of LFA strips. Therefore, it is a wise strategy to initially reduce GO and then use it as an LFA label. The reduction step was

performed by a green reductant. The color of GO solution changed to black following a 15 min reduction process (Fig. 2a), demonstrating enhanced light absorption in the visible light spectrum (~450-850 nm) owing to the restoration of conjugated π network of graphene sheets. Moreover, UV-vis spectra of GO and rGO clearly confirms the change in peak position of GO from 230 nm to 260 nm after the reduction process (Fig. 2a) [23]. The prepared rGO flakes were freely dispersible in DI water without aggregation due to mild reduction under controlled conditions. Figure 2b is the UV-Vis spectra of conjugated GO as well as conjugated rGO. UV-Vis spectra of conjugated GO shows an absorbance peak at 280 nm that corresponds to the absorbance peak of proteins confirming the presence of antibodies with rGO. However, UV-Vis spectra of conjugated rGO shows a peak at 260 nm (Fig. 2b). It appears that the peak of proteins at 280 nm is overlapped with the main peak of rGO at 260 nm, probably due to closeness of the position of 260 and 280 nm peaks.

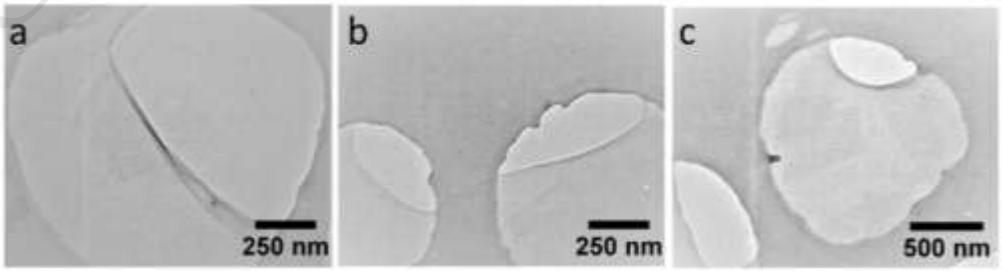

Fig. 1. TEM images of single layer GO (a), rGO (b) and mAb-conjugated rGO (c).

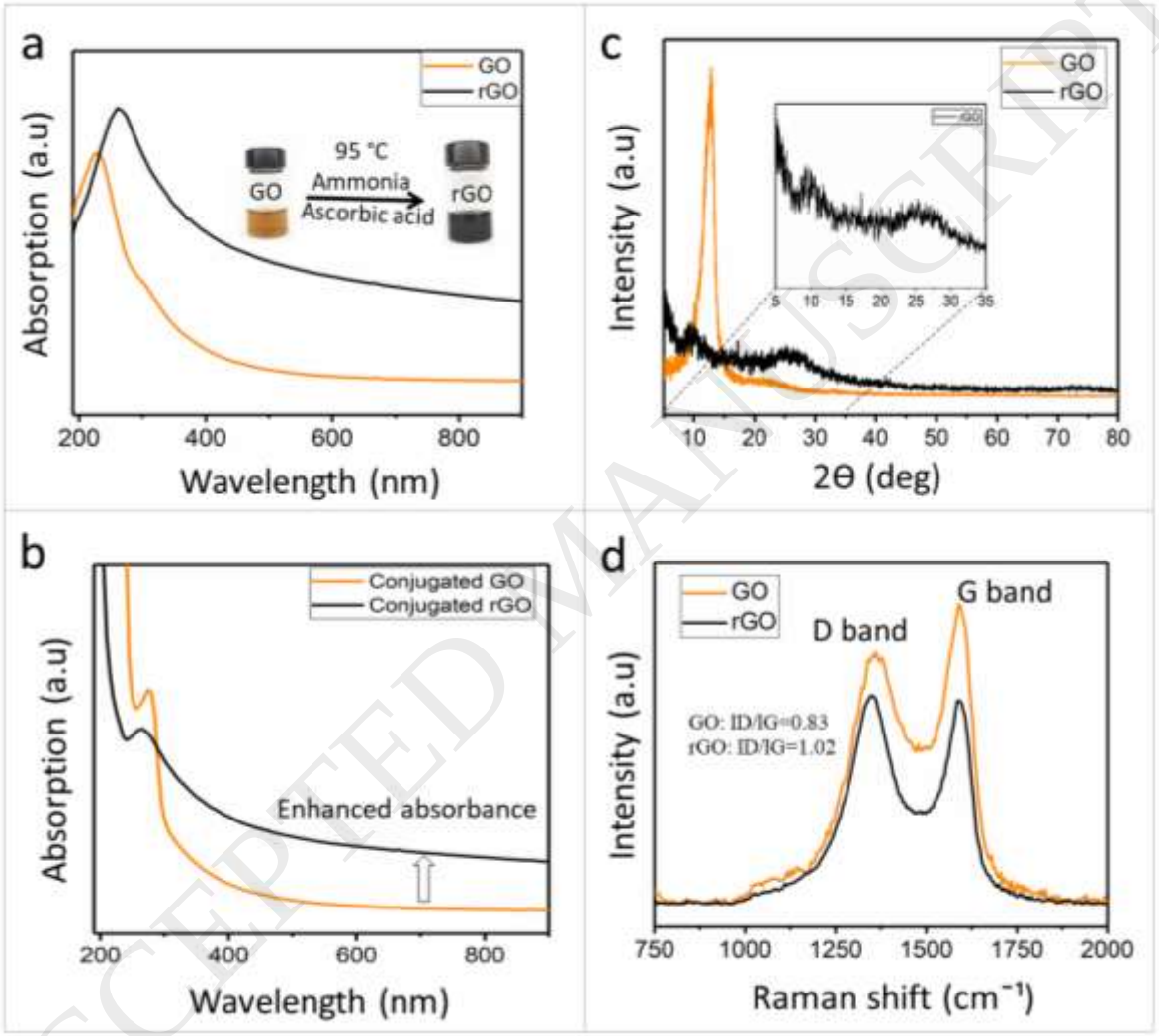

Fig. 2. (a) UV-Vis spectra of GO and rGO. (b) UV-Vis spectra conjugated GO and conjugated rGO, inlet shows synthesis process of rGO. (c) XRD spectra of GO and rGO indicating characteristic peak of GO in comparison to rGO. (d) Raman spectra of GO and rGO showing D and G bands of these materials.

To further characterize GO and rGO utilized in this study, XRD and Raman characterization were used. XRD pattern of graphite as the raw material of GO has only one main characteristic peak at about 27° (2Θ) with crystal orientation of 002, which is attributed to a 3.36 Å interlayer space. During the oxidation of graphite to GO, this peak disappears and characteristic peak of GO appears in a position at around 10° (2Θ) [24]. XRD pattern of GO shows that the characteristic peak of GO is at 12.8° that corresponds to an interlayer space of 8.02 Å (Fig. 2c). The change in peak position (from 27° in graphite to 12.8° in GO) and interlayer space (from 3.36 Å in graphite to 8.02 Å in GO) relates to functional groups and remaining water molecules in interlayer galleries of GO [24]. Figure 2c also shows XRD pattern of rGO confirming the disappearance of GO peak at 12.8°. The disappearance of this peak after reduction of GO by ascorbic acid, confirms successful reduction of GO to rGO [25].

Raman spectroscopy is an appropriate technique to study structures of carbon based materials including graphene derivatives. The characteristic peaks in Raman spectroscopy for carbon materials are D and G bands (located at 1350 and 1580 cm^{-1}), which are respectively related to the local defects and the sp_2 structure of

graphene derivatives. Figure 2d shows Raman spectra of GO and rGO. The D to G ratio (ID/IG) is 0.83 for GO and 1.02 for rGO. This change of ID/IG demonstrates successful reduction of GO [26].

3.2. Basic operation principle of LFAs

LFA tests usually have a strip format with a width of 4-6 mm and a length of 60-70 mm. They are composed of four main parts assembled on a plastic support as follows (Fig. 3a): Sample pad: it is where the sample is added to the test strip and usually made of cellulose. Conjugate pad: it is made of glass fiber and is where the conjugated labels are added manually or sprayed with a spraying machine. Detection pad: It is where visual signal appears based on the presence of a target analyte in the sample. Detection pad is a layer of nitrocellulose membrane with different pore sizes, usually 5, 8 or 15 µm, in which two lines of capture molecules known as test zone (T.Z) and control zone (C.Z) are printed by a dispensing machine. Absorption pad: as a reservoir made of cellulose or cotton, it is where the running sample is collected from the test strip. In addition to these parts of a standard LFA test strip, there may be other parts added to a strip such as red blood cell filters that are used as sample pads to remove red blood cells from blood samples. In LFA strips, the sample pad is contacted with a sample potentially

having a target analyte that drives the sample through the LFA test strips by capillary forces. When the sample reaches the conjugate pad, the conjugated labels are mixed with the sample and bound to the analyte, if present. On the detection pad, the sample and labels pass through T.Z and C.Z and are finally collected within the absorbent pad. The appearance of a visual signal indicates the presence of an analyte in the sample and depends on the format of assay used in LFA strips. There are two forms of assay for LFA technology, i.e. comparative and sandwich assays. In a competitive assay, the target analyte and the conjugated label compete to be captured on T.Z, resulting in a visual color inversely proportional to the amount of analyte. In a sandwich format, the complex of analyte and conjugated label is linked to the T.Z producing a visual color directly proportional to the analyte concentration. In both types of assays, a high density color appears on the C.Z confirming that the assay works properly. For multiple analyte detection, several test lines are printed on the detection pad; consequently there are multiple conjugated labels added to the conjugate pad corresponding to each target analyte [2, 3]. In this work, a sandwich format was used, in which T.Z and C.Z were manually printed as circular dots (Fig. 3a).

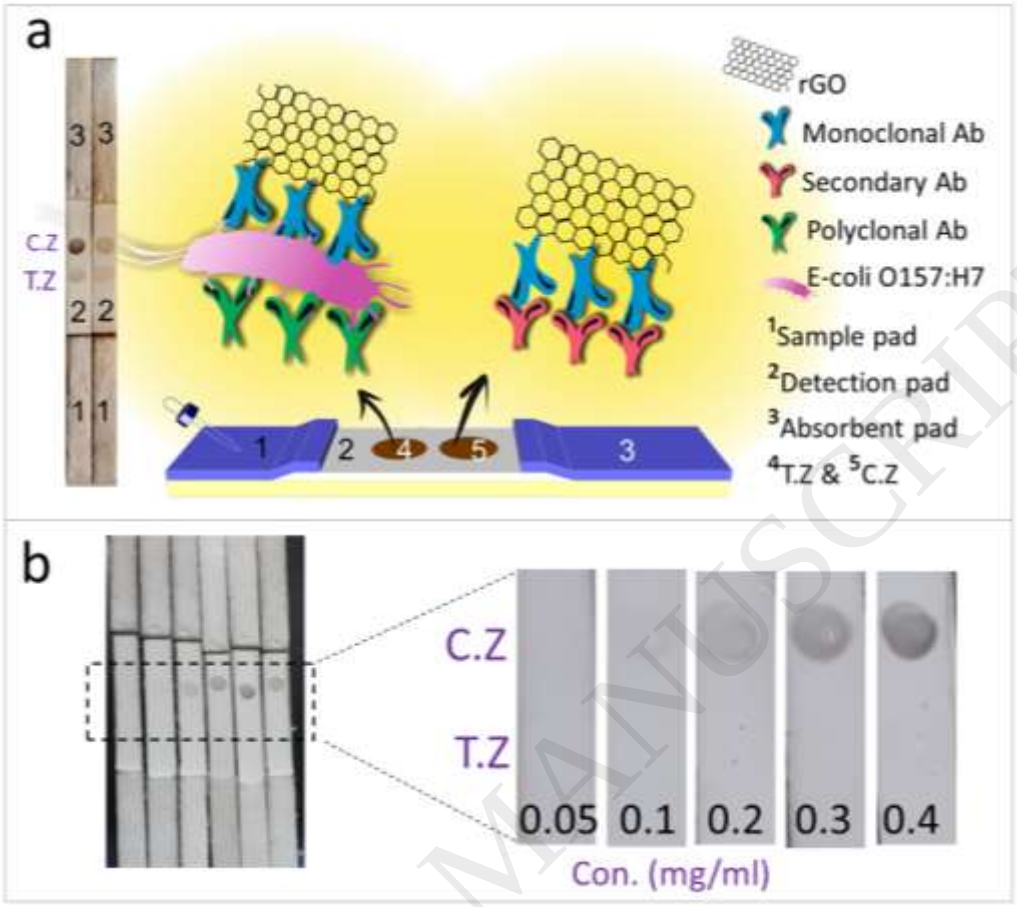

Fig. 3. (a) Schematic view of rGO-based lateral flow immunoassay in sandwich format for E. coli O157:H7 detection. The digital images are LFA strips with rGO (left strip) and GO (right strip). (b) LFA test strips with rGO-based labels conjugated to various amounts of anti E. coli O157:H7 monoclonal antibody: (C.Z is Control Zone and T.Z is Test Zone).

3.3. Conjugation

GO and rGO were conjugated to anti E. coli O157:H7 monoclonal antibody using a physical absorption approach. To optimize amount of mAb for functionalization of rGO, various concentrations of mAb (0.05, 0.1, 0.2, 0.3, 0.4 mg/ml) were added to suspensions of rGO. The prepared rGO-mAb conjugates were added to LFA strips having both control and test zones. Figure 3b shows that the signal intensity of control zones is enhanced with increasing the concentration of E. coli O157:H7 monoclonal antibody. Therefore, to provide the best signal intensity, the optimum concentration of mAb conjugated to rGO was selected to be 0.4 mg/ml per mg of rGO. This concentration was also used for conjugation of GO to mAb. Figure 3b also indicates that there is no signal intensity of unspecific interactions on the test zones of the test strips. This strongly confirms that the black color observed on the control zones are derived from specific interactions between mAb conjugated to rGO and anti IgG deposited on the control zone. Figure 3 also demonstrates successful conjugation of mAb to rGO.

3.4. Evaluation of GO and rGO-based labels for detection of pure E. coli O157:H7

To determine the visual limit of detection (LOD) of GO and rGO-based LFA strips, a sample preparation step was used. Various concentrations of E. coli O157:H7 (from

~10^8 CFU/mL to ~10^3 CFU/mL) in running buffer with a final volume of 95 µL were mixed with 5 µL GO-mAb or rGO-mAb conjugates. Then, each strip was placed within an ELISA well containing this mixture. The capillary forces caused the mixture to move through the length of LFA strips. The visual signal of test zones were read after 20 minutes. Figure 4 indicates that the intensity of test zones increases with increasing analyte concentration demonstrating functionality of the test strips having GO and rGO as visual labels for detection of E. coli O157:H7. Figure 4a also shows that the intensity of control zones decreases with increasing the bacteria concentrations. This is because high concentration of analyte consumes the majority of GO and rGO labels within the test zone and a small amount of the labels spreads to the control zone producing a lower visual signal in test strips with higher concentrations of bacteria. Figure 4a and 4b show that the LOD of rGO and GO-based strips were ~10^5 CFU/mL, which is comparable to LOD of conventional gold nanoparticles [19].

Figure 4 shows that the visual signal intensity of rGO-based strips is much higher than GO-based strips as seen by naked eye. To confirm this quantitatively, a digital image of the strips with ~10^5 CFU/mL of E. coli O157:H7 was converted to a binary image by ImageJ software. Figure 4d is a comparison between quantitative color intensity of C.Z and T.Z of test strips shown in figure 4c, demonstrating that rGO-

based label has higher intensity than GO-based label. This is predicted from UV-Vis spectra of GO and rGO owing to the enhanced light absorption of rGO compared with GO of the same concentrations.

The calibration curve of rGO-based strips was prepared by plotting the IT/IC against the logarithm of standard concentrations of E. coli O157:H7 (0, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 CFU/ml). Figure 4e indicates the standard cure having a linear range from 10^4 to 10^7 CFU/ml with a correlation coefficient of R^2=0.95 and a regression equation of y=0.32x-1.23, where y is IT/IC and x is the E. coli O157:H7 concentration. Error bars were based on three duplicate measurements at each E. coli O157:H7 concentration.

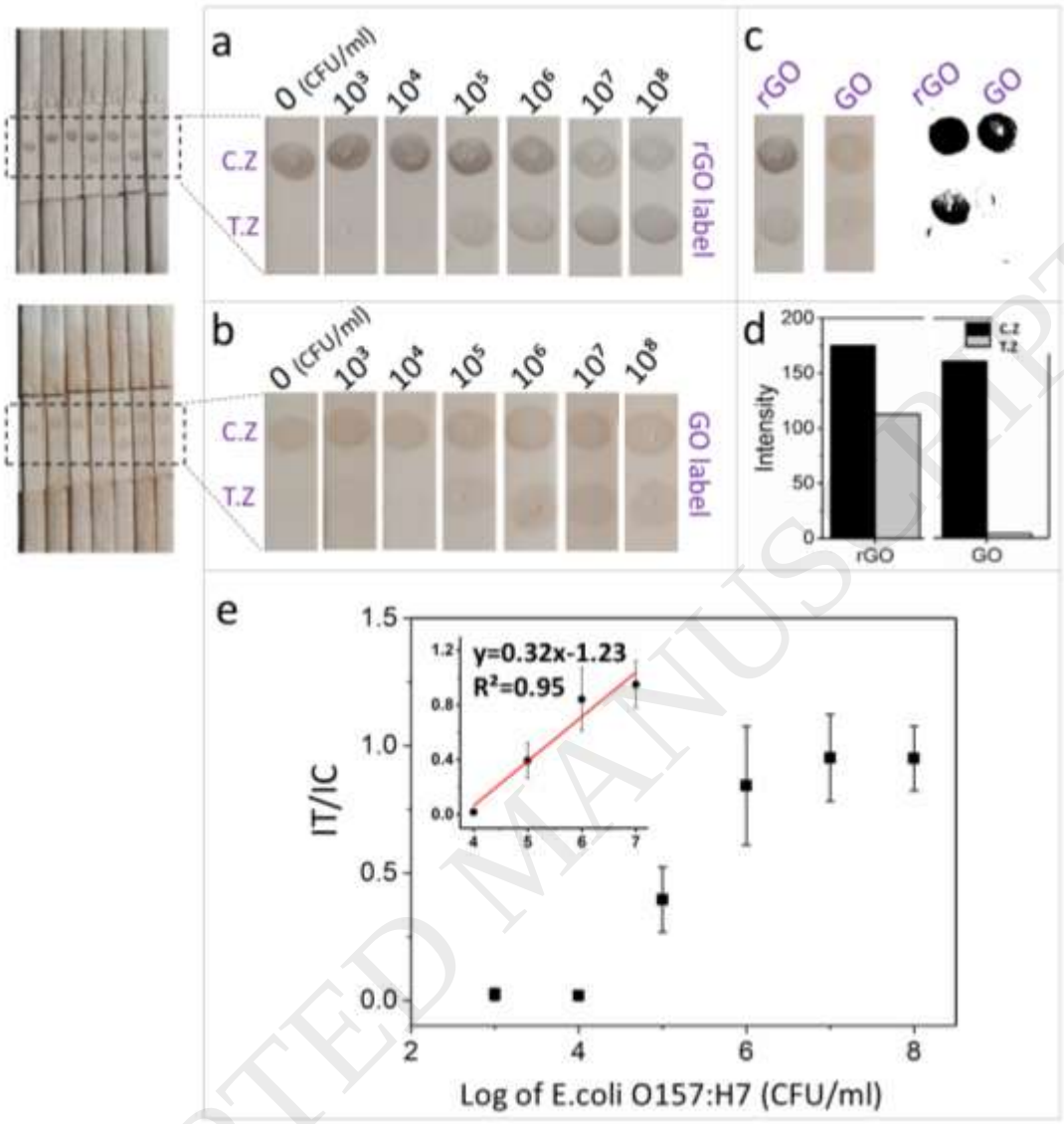

Fig. 4. (a) LFA test strips with rGO labels after assay of different concentrations of pure E. coli O157:H7. (b) LFA test strips with GO labels after assay of different concentrations of pure E. coli O157:H7. (c) LFA test strips with rGO and GO labels after assay of ~10^5 CFU/mL of pure E. coli O157:H7 as well as their corresponding binary image processed by ImageJ software. (d) Quantitative results of test and control zones of (c). (e) Colorimetric calibration curve of rGO-based strips for

standard concentrations of the target bacteria, obtained by plotting IT/TC against the logarithm of E.coli O157:H7 concentrations. (C.Z: Control Zone and T.Z: Test Zone).

3.5. Evaluation of rGO-based labels in real samples

In order to demonstrate efficiency of the LFA strips with rGO-based labels in real food samples, milk and drinking water were directly spiked with different concentrations of E. coli O157:H7. To produce 10-fold dilution of the real samples, 1 ml of each sample was diluted with 9 ml running buffer, from which samples with final concentrations from ~10^8 CFU/mL to ~10^3 CFU/mL were prepared.

Figure 5a shows that the strips are applicable for the detection of the target bacteria in real samples within 20 minutes with a visual LOD of ~10^7 CFU/mL for milk that is two orders of magnitude higher than LOD of the rGO-based labels in standard solutions of bacteria in PBS. It should be noted that the strip with ~10^6 CFU/mL of the target bacteria also shows a weak intensity showing that the LOD of rGO-based LFA strips is lower than ~10^7 CFU/mL. The decreased sensitivity of the strips in milk can be attributed to interferences caused by unidentified compounds of the milk that can interfere with immunochemical interactions between the

target bacteria and rGO labels. The visual LOD for drinking water was 10^5 CFU/mL, which is better that the LOD for milk. This is because drinking water has less interfering compounds compared with highly complex matrix of milk.

In order to evaluate the accuracy and precision of the rGO based strips in this study, recovery studies were conducted by analyzing spiked milk and drinking water with ~10^6, ~10^7 and ~10^8 CFU/ml of E. coli O157:H7. The analysis was completed with three replicates at each spikes concentration (Table 2). The average recoveries were from 55 to 103.25%, with a CV ranging from 5.33 to 13.03%. In comparison to the standard method of plate culture and colony counting as the reference method, which lasts at least 1 day, the rGO based detection described in this study takes only 20 minutes. Although the colony counting is more accurate and sensitive, the method described herein is fast, simple, portable and doable by ordinary people.

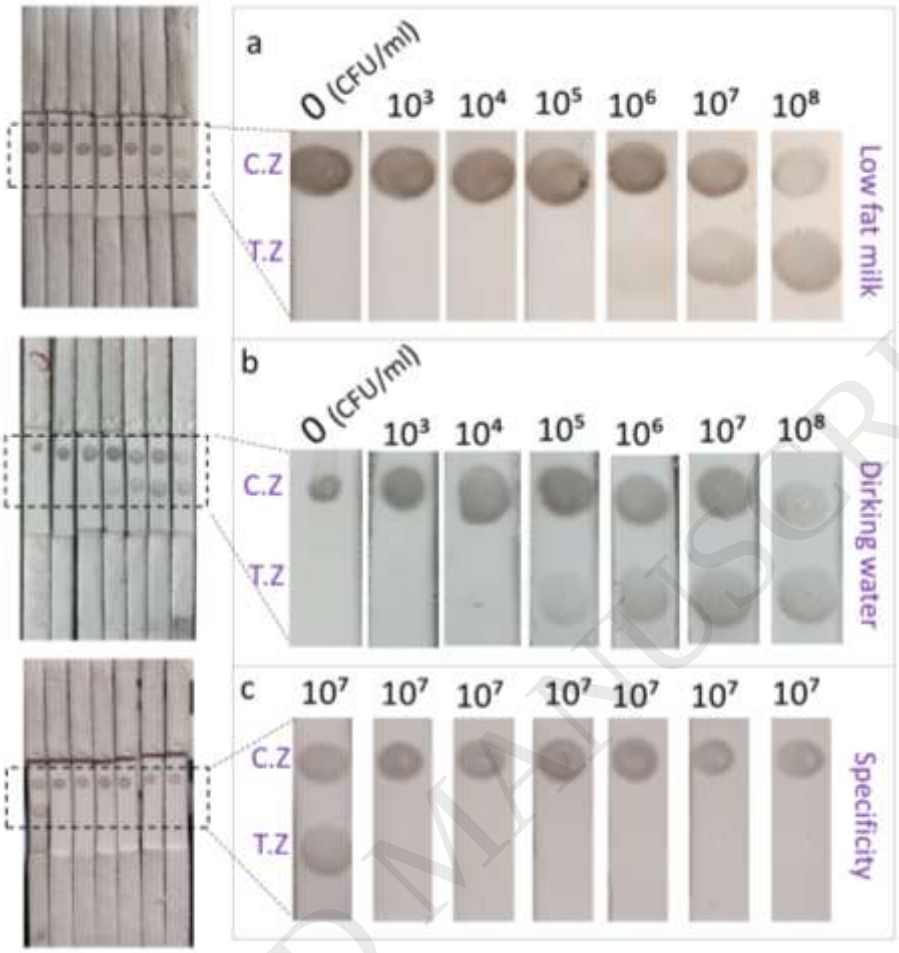

Fig. 5. (a) LFA test strips with rGO labels after assay of different concentrations of E. coli O157:H7 (~10^8 CFU/mL to ~10^3 CFU/mL) spiked in milk. (b) LFA test strips with rGO labels after assay of different concentrations of E. coli O157:H7 (~10^8 CFU/mL to ~10^3 CFU/mL) spiked in milk. (c) Evaluation of specificity of the strips with rGO-based labels on 6 types of isolated strains (~10^7 CFU/mL): from left to right E. coli O157:H7, E. coli O20, E. coli ATCC 25922, Bacillus subtilis ATCC 19659, Staphylococcus epidermidis ATCC 12228, Pseudomonas aeruginosa ATCC 27853 and Salmonella typhimurium ATCC 14028. (C.Z: Control Zone and T.Z: Test Zone).

Tab. 2. Precision and stability of the rGO based test strips in spiked samples of milk and drinking water.

Food material	*Spiked E. coli O157:H7(log CFU/mL)*	*Detected E. coli O157:H7(log CFU/mL)*[a]	*Recovery%*	*SD*	*CV%*
Milk	8	8.26	103.25	0.65	7.86
	7	6.56	93.71	0.35	5.33
	6	3.3	55	0.43	13.03
Drinking water	8	7.93	99.12	0.45	5.67
	7	6.83	97.57	0.85	12.44
	6	5.66	94.33	0.66	11.66

[a]*Mean of three determinations. CV=SD/mean*

3.6. Specificity of strips

To evaluate specificity of the strips with rGO-based labels, 6 types of isolated strains(10^7 CFU/mL): E. coli O20, E. coli ATCC 25922, Bacillus subtilis ATCC 19659, Staphylococcus epidermidis ATCC 12228, Pseudomonas aeruginosa ATCC 27853 and Salmonella typhimurium ATCC 14028 (figure 5b) were tested as negative controls by the LFA strips. Figure 5c shows that no perceivable signal was recognized on the test zones of the LFA strips when a bacterial strain other that

the target bacteria was applied. Therefore, the LFA strips detect only E. coli O157:H7 strain and has no cross reactivity with other bacterial strains.

4. Conclusion

In this work, rGO was introduced as a new visual label for lateral flow immunoassay. It was confirmed that rGO can provide high color intensity for the detection of E. coli O157:H7 owing to enhanced light absorption after reduction of GO. Limit of detection of rGO for the target bacteria detection was ~10^5 (CFU/ml) for standard bacteria solution, which is comparable to gold nanoparticles, a widely used label. Our results indicate that rGO is highly capable of being used as a low price visual label for E. coli O157:H7 and a wide variety of other analytes.

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Author Contributions

All authors have given approval to the final version of the manuscript.

The authors declare that they have no conflict of interest.

This work was supported by Department of Medical Nanotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran.

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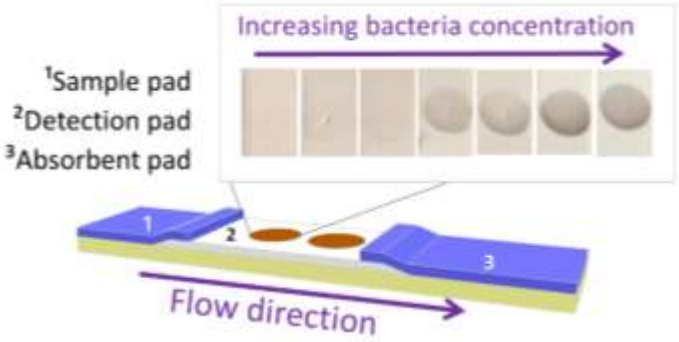
Graphical abstract



Tables and figures captions:

Tab. 1. Comparison of rGO label with some other LFA labels.

Tab. 2. Precision and stability of the rGO based test strips in spiked samples of milk and drinking water.

Fig. 1. TEM images of single layer GO (a), rGO (b) and mAb-conjugated rGO (c).

Fig. 2. (a) UV-Vis spectra of GO and rGO. (b) UV-Vis spectra conjugated GO and conjugated rGO, inlet shows synthesis process of rGO. (c) XRD spectra of GO and rGO indicating characteristic peak of GO in comparison to rGO. (d) Raman spectra of GO and rGO showing D and G bands of these materials.

Fig. 3. (a) Schematic view of rGO-based lateral flow immunoassay in sandwich format for E. coli O157:H7 detection. The digital images are LFA strips with rGO (left strip) and GO (right strip). (b) LFA test strips with rGO-based labels conjugated to various amounts of anti E. coli O157:H7 monoclonal antibody: (C.Z is Control Zone and T.Z is Test Zone).

Fig. 4. (a) LFA test strips with rGO labels after assay of different concentrations of pure E. coli O157:H7. (b) LFA test strips with GO labels after assay of different concentrations of pure E. coli O157:H7. (c) LFA test strips with rGO and GO labels after assay of ~10^5 CFU/mL of pure E. coli O157:H7 as well as their corresponding binary image processed by ImageJ software. (d) Quantitative results of test and control zones of (c). (e) Colorimetric calibration curve of rGO-based strips for standard concentrations of the target bacteria, obtained by plotting IT/TC against the logarithm of E.coli O157:H7 concentrations. (C.Z: Control Zone and T.Z: Test Zone).

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