Hairpin-structured probe conjugated nano-graphene oxide for the cellular detection of connective tissue growth factor mRNA

Wang, Min; Wiraja, Christian; Wee, Meiling; Hu, Lianzhe; Xu, Chenjie; Yeo, David Chen Loong

2018

Wang, M., Wiraja, C., Wee, M., Yeo, D. C. L., Hu, L., & Xu, C. (2018). Hairpin-structured probe conjugated nano-graphene oxide for the cellular detection of connective tissue growth factor mRNA. *Analytica Chimica Acta*, 1038, 140-147. doi:10.1016/j.aca.2018.07.016

<https://hdl.handle.net/10356/90104>

<https://doi.org/10.1016/j.aca.2018.07.016>

© 2018 Elsevier B.V. All rights reserved. This paper was published in *Analytica Chimica Acta* and is made available with permission of Elsevier B.V.

Downloaded on 26 Apr 2023 07:52:44 SGT

Hairpin-structured probe conjugated nano-graphene oxide for the cellular detection of connective tissue growth factor mRNA

Min Wang^{1,2*}, Christian Wiraja², Meiling Wee³, David Yeo², Lianzhe Hu^{4*}, Chenjie Xu^{2*}

1. School of Pharmaceutical Sciences, Chongqing University, Chongqing 401331, China
2. School of Chemical and Biomedical Engineering, Nanyang Technological University, 70 Nanyang Drive, Singapore 637457, Singapore
3. School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551, Singapore
4. Chongqing Key Laboratory of Green Synthesis and Applications, and College of Chemistry, Chongqing Normal University, Chongqing 401331, China

Email: wang_min@cqu.edu.cn; lianzhehu@cqu.edu.cn; cjxu@ntu.edu.sg

Abstract

Identification of abnormal scars at their early stage has attracted increasing attentions as the scars can only be assessed qualitatively and subjectively upon maturity, when no invasive procedure is involved.

Methods: This report introduces a fluorescent probe that targets a potential abnormal scar biomarker (connective tissue growth factor (CTGF) mRNA) in skin fibroblasts. This probe is constructed of hairpin-structured probes (HPs) targeting CTGF mRNA and the nano-graphene oxide (nano-GO) base. The HPs are non-covalently absorbed on the surface of nano-GO, which pre-quenches the fluorescence of HPs. Close proximity of complementary CTGF mRNA would lead to preferential HP hybridization and dissociation from nano-GO, which restores the fluorescence signal from HPs.

Results: The nano-GO/CTGF probe can quantitatively and specifically recognize the target sequence of CTGF mRNA in the range of 0-200 nM with the detection limit of 0.7 nM. This probe showed good stability under nuclease degradation. Higher fluorescence signal from hypertrophic scar fibroblasts (HSF) was observed compared to normal dermal fibroblasts (NDF). During the treatment of HSF with anti-scarring drugs, significant decrease in CTGF mRNA expression was observed.

Conclusions: The nano-GO-based nanoprobe allows us to distinguish abnormal fibroblasts derived

from abnormal scars and assess the effectiveness of anti-scarring drugs like Repsox and transforming growth factor-beta type I receptor (TGF- β RI) siRNA.

Key words:

abnormal scar, skin fibroblast, nucleic acid probe, graphene oxide, mRNA

Introduction

Abnormal scars including hypertrophic scars and keloids exhibit distinct clinical characteristics in scar color, thickness, and pliability, which enables clinicians to assess and classify the scars through Vancouver Scar Scale.[1] However, this scar assessment is qualitative, subjective and resource-intensive, and only applicable for mature scars rather than newly-formed scars. Moreover, abnormal scars are traditionally treated by surgical excision when they are mature, which is associated with high recurrence rates.[2] Fortunately, early diagnosis of abnormal scars can be beneficial to scar prevention and treatment prior to excessive formation of fibrosis tissue.[3] Recent researches show that the population of fibrocytes (*i.e.* the precursors of fibroblasts) in abnormal scar tissue was higher than that in normal scar tissue, and thus may be an early index for distinguishing abnormal scars.[4,5] Nevertheless, identification of fibrocytes involved the invasive and time-consuming cryosectioning and immunostaining with expensive antibodies. To address this issue, potent strategies for non-invasively identifying the abnormal scars at their early stage are urgently needed.

mRNA has attracted growing interests as the biomarkers for the early disease diagnosis.[6,7] It could be examined through northern blotting[8] and polymerase chain reaction (PCR)[9] and probe-assisted fluorescence imaging[10]. The fluorescence imaging based on nucleic acid probes has been considered as a non-invasive approach for providing both temporal and spatial distribution of mRNA at the cellular level in a quantitative way.[11,12] Transforming growth factor-beta type I (TGF- β I) has been proven to be responsible for the fibrotic scarring.[13] Moreover, connective tissue growth factor (CTGF) mRNA, a TGF- β I downstream mediator, was shown to be overexpressed in fibroblasts from abnormal scars, and its down-regulation through TGF- β I inhibitor can significantly limit abnormal scarring progression.[14-16] This finding enables CTGF mRNA to be a potential biomarker for identifying abnormal scar fibroblasts.

Recently, we have demonstrated that CTGF mRNA can be monitored with spherical-nucleic-acids in hypertrophic scar fibroblasts as well as in a pre-clinical rabbit ear wound model, which enabled the visual and spectroscopic quantification of abnormal fibroblasts in the dermis.[17]

Nano-graphene oxide (nano-GO) nanosheets, a derivative of two-dimensional graphene, have received considerable attention as gene carriers for theranostics owing to their excellent properties including high water dispersibility, low cytotoxicity, easy functionalization and high fluorescence quenching capability.[18] nano-GO also shows high affinity to single-stranded oligonucleotide probes through the non-covalent interaction. Once inside a cell, nano-GO can protect the absorbed probes from nuclease unspecific digestion.[19] Moreover, the excellent fluorescence quenching ability of nano-GO would provide a low background and thus enable the sensitive response to analytes.[20] Although nano-GO has been applied for many applications such as apoptosis monitoring[21], cancer diagnosis[22], and induced pluripotent stem cell generation[23], it has not been adapted for in skin cell identification.

This article introduces a nano-GO-based mRNA probe for monitoring CTGF mRNA expression in fibroblasts. This probe is constructed with a hairpin-structured probe (HP) targeting CTGF mRNA and nano-GO. The HPs are non-covalently absorbed on the surface of nano-GO due to the weak interaction between graphene aromatic plane and nucleobases. Nano-GO quenches the fluorescence of the dye on HP due to fluorescence resonance energy transfer (FRET) effect. The presence of the CTGF mRNA induces the formation of double-stranded oligonucleotides between HP and CTGF mRNA, which desorbs HPs from the nano-GO surface and restores the fluorescence of the dye on HPs (Scheme 1a). This probe can specifically recognize the target sequence of CTGF mRNA as low as 0.7 nM. It allows the identification of hypertrophic scars from normal fibroblasts. Furthermore, we can use it to assess the treatment effectiveness of the anti-scarring drugs like Repsox and specific siRNA through targeting TGF- β I (Scheme 1b).

Scheme 1. Schematic illustration of the working mechanism of the nano-GO/HP probe for CTGF mRNA detection. (a) Complexation and de-complexation of the probe; (b) Assessment of the effectiveness of anti-scarring drugs with the probe.

Methods and Materials

Graphite powder (99.8%, 325 mesh) was obtained from Alfa-Aesar. Repsox and all the oligonucleotides listed in [Table S1](#) were purchased from Sigma-Aldrich (Singapore). Dulbecco's modified eagle medium (DMEM) with L-glutamine and 4.5 g/L D-glucose, fetal bovine serum (FBS), penicillin-streptomycin (10,000 U/mL), and trypsin-EDTA (0.5%) were obtained from Gibco Life Technologies (Singapore). Hoechst 33342 and Lipofectamine® 2000 were obtained from Thermo Fisher Scientific (Singapore). DNase I (1,000 U/mL) and reverse transcription kit containing M-MLV RNase H (-) Mutant and dNTP were purchased from Promega (Singapore). iQ SYBR Green Supermix was obtained from Bio-Rad (Singapore). Phosphate buffered saline (PBS, 1X) was purchased from Lonza (Singapore). All reagents were of analytical reagent grade and used without further purification.

Dynamic light scattering (DLS) measurement was performed on a Zetasizer Nano ZS (Malvern Instruments, UK). UV-vis spectrum was measured on a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Fluorescence spectra were collected on a LS55 Fluorescence Spectrometer (Perkin Elmer, USA). Atomic force microscopy (AFM) images were

obtained by a MFP-3D atomic force microscope (Asylum Research, USA). All the fluorescence imaging was performed on an LX71 inverted fluorescence microscope (Olympus, Japan). Flow cytometry data were obtained on an LSR Fortessa X-20 flow cytometer (BD Biosciences, USA) by collecting at least 100,000 gated events.

Construction of the probe and test of its specificity and sensitivity

Nano-GO sheets were synthesized from graphite powders according to Hummer's method[24] and dispersed in water with a final concentration of 1 mg/mL. Then, 100 nM of Cy3-labeled CTGF HPs in PBS (10 mM, pH 7.4) was incubated with 10 µg/mL of nano-GO solution in dark for 5 min to form the nano-GO/HP probe.

For the sensitivity test, the nano-GO/HP probe was mixed with different concentrations of target sequence in the range of 0-1 µM. After incubation at room temperature for 40 min, the fluorescence spectra of samples upon excitation at 535 nm was recorded and the fluorescence intensity at 560 nm was analyzed. For the specificity test, the nano-GO/HP probe was incubated with 200 nM of different mismatched (i.e. single-base mismatched, two-bases mismatched and three-bases mismatched) target sequences for 40 min, followed by the measurement of the fluorescence intensity at 560 nm under excitation at 535 nm.

Nuclease resistance

The nano-GO/HP probe and 100 nM of CTGF molecular beacon (MB, i.e., HP probe labeled with Black Hole Quencher 2 (BHQ2) at the 3' end) were incubated at 37°C in the absence and presence of 2 U/mL DNase I. At different time points, the fluorescence intensity of samples at 570 nm was recorded upon the excitation at 550 nm by using a SpectroMax M5 plate reader (Molecular Devices, USA).

Monitoring of CTGF mRNA expression in fibroblasts

Fibroblasts derived from normal human dermis (NDF) and human hypertrophic scars (HSF) were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C with 5% CO₂. 24 hours before the experiments, NDF and HSF were seeded onto 12-well plates with the same cell density (1×10^5 cells). Then cells were incubated with 10 µL of the probe solution in 1 mL of DMEM supplemented with 1% FBS and 1% penicillin-streptomycin. After incubation for 8 hrs, the cells were washed with PBS three times and replenished with fresh normal culture medium. Later cells was examined by fluorescence microscopy, flow cytometry and quantitative

reverse transcription polymerase chain reaction (RT-qPCR).

In RT-qPCR analysis, the cellular mRNA was extracted from HSF and NDF by using QIAGEN RNA kit.[12] The expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal reference. The values of $2^{-\Delta(C_T(CTGF) - C_T(GAPDH))}$ for all the samples were used for evaluating the fold change of CTGF mRNA relative to GAPDH mRNA. The statistical significance of CTGF mRNA expression between NDF and HSF was determined by calculating the *p* value using one-way analysis of variance (ANOVA).

Assessment of the effectiveness of anti-scarring drugs

In the Repsox treatment, HSF cells were seeded onto 12-well plates until the confluency reached 90%. Then cells were incubated with 65 μ M of Repsox in DMEM supplemented with 1% FBS and 1% penicillin-streptomycin. After incubation for 48 hrs, the cells were washed with PBS three times to remove the excess Repsox.

In the transforming growth factor-beta type I receptor (TGF- β RI) siRNA treatment, DMEM containing 1% penicillin-streptomycin (serum-free medium) was used for preparing siRNA-lipofectamine assembly. Briefly, three types of TGF- β RI siRNA duplexes covering different regions of the human TGF- β RI mRNA (i.e. TGF- β RI siRNA1, TGF- β RI siRNA2 and TGF- β RI siRNA3 in [Table S1](#)) were mixed at the molar ratio of 1:1:1. Then 50 μ L of serum-free medium containing 24 μ M TGF- β RI siRNA mixture was incubated with 50 μ L of serum-free medium containing 6 μ L Lipofectamine® 2000 reagent for 10 min at room temperature. Before the gene silence experiment, HSF cells were seeded onto 12-well plates until the confluency reached 90%. The cells were transfected with the as-prepared siRNA-lipofectamine assembly (i.e. 40 pmol siRNA and 2 μ L Lipofectamine® 2000 per well) in DMEM supplemented with 1% FBS and 1% penicillin-streptomycin. After incubation for 48 hrs, the cells were washed with PBS three times to remove the excess assembly.

To quantify the CTGF mRNA expression, the above treated cells were labeled with the probe according to the above protocol. For nuclei staining, HSF were incubated with 5 μ g/mL of Hoechst 33342 in PBS at room temperature for 10 min. The excess Hoechst 33342 was removed by washing cells with PBS. The fluorescence of cells was examined by fluorescence microscopy and flow cytometry. The cellular mRNA was also quantified with RT-qPCR as mentioned above. The statistical significance of CTGF mRNA expression in HSF with and without Repsox or

siRNA treatment was determined by calculating the p value using one-way analysis of variance (ANOVA).

Results

Synthesis and characterization of nano-GO nanosheets

The typical wrinkled morphology of graphene sheets was observed from nano-GO by TEM characterization (Figure 1a). AFM result revealed that it exhibited a uniform height of *ca.* 1.1 nm (Figure 1b), indicating its single-layer structure.[25] The nano-GO had a hydrodynamic diameter of 534 ± 96 nm (Figure 1c) and was negatively charged with a zeta potential of -27.9 ± 12.3 mV (Figure S1). The negative charge was due to the presence of abundant oxygen-containing groups on the surface, which also permitted nano-GO to disperse well in aqueous solution. The solution containing nano-GO exhibited a wide UV-vis absorption below 700 nm with a strong band at 230 nm and a shoulder around 300 nm (Figure 1d), which suggests that nano-GO can be a potential fluorescence quencher.

Figure 1. Characterization of the nano-GO sheets with (a) TEM, (b) AFM, (c) DLS, and (d) UV-vis.

Preparation and examination of nano-GO/HP probe

HPs were first designed for specifically recognizing the homo sapiens CTGF mRNA with an E value of 0.045 analyzed by using Basic Local Alignment Search Tool (BLAST). They were absorbed on the surface of nano-GO sheets *via* noncovalent interactions including π - π stacking, hydrogen bonding, hydrophobic interaction, and van der Waals forces, forming the nano-GO/HP probe assembly.[21,22,19] The sensing principle of the assembly was based on the two characteristics of nano-GO sheets: 1) the high fluorescence quenching efficiency of nano-GO sheets; 2) the distinct difference on the affinity of nano-GO sheets towards single-stranded and double-stranded DNA.[18]

The fluorescence quenching was confirmed by monitoring the fluorescence change of the HP solution with the continuous addition of nano-GO. As shown in [Figure S2](#), the fluorescence intensity decreased gradually with the increasing concentration of nano-GO sheets in a range of 1-20 $\mu\text{g/mL}$. The corresponding fluorescence quenching efficiency (QE) was calculated according to the formula $\text{QE} = (F_0 - F)/F_0 \times 100\%$ (F_0 and F represents the fluorescence intensity at 560 nm without and with the addition of nano-GO, respectively). As shown in [Figure S2b](#), the QE of nano-GO increased with its concentration and reached a maximum of 98.8% when the nano-GO concentration was 20 $\mu\text{g/mL}$. In addition, the quenching dynamic study revealed that the absorption of HPs onto the nano-GO sheets completed within 5 min ([Figure S3](#)).

We then studied the probe response to target sequence. As shown in [Figure 2a](#), there was no fluorescence from the nano-GO/HP probe solution. However, upon the addition of target sequence, there was a 58-fold enhancement in the fluorescence intensity. Clearly, this was due to the detachment of HP from nano-GO surface after the hybridization of HP and the complementary target sequence. The fluorescence restoration took ~ 35 mins post the addition of the target sequence ([Figure S4](#)). We further studied the sensitivity of the probe by examining the fluorescence restoration under the presence of various concentrations of target sequence ([Figure 2b & 2c](#)). The probe allowed the identification of target sequence as low as 0.7 nM. The linear range between fluorescence and concentration of target sequence was found between 5 nM to 200 nM. These results demonstrate the potential ability of the nano-GO/HP probe to quantify the intracellular CTGF mRNA with excellent sensitivity.

Figure 2. Complexation of nano-GO/HP probe and target sequence: (a) Fluorescence spectra of 20 $\mu\text{g/mL}$ nano-GO (black), 100 nM HP (red), the nano-GO/HP probe (20 $\mu\text{g/mL}$ nano-GO and 100 nM CTGF HP) in the absence (green) and presence (blue) of 1 μM target sequence. (b) Fluorescence spectra of the nano-GO/HP probe (10 $\mu\text{g/mL}$ nano-GO and 100 nM HP) in the presence of different concentrations of target sequence (a-i: 0, 10, 30, 50, 100, 150, 200, 400, 800 nM). (c) Plots of fluorescence intensity at 560 nm versus the concentration of target sequence. (d) Linear relationship between the fluorescence intensity at 560 nm and the target concentration in a range of 5-200 nM.

In addition, the mismatched target sequences were employed to examine the specificity of the designed HPs for CTGF mRNA recognition. **Figure 3** shows the distinct fluorescence responses of the probe to target sequences at the same concentrations with and without mismatched bases. All the mismatched targets containing one to three mismatched bases showed lower fluorescence signals than the perfectly matched target DNA, which can be ascribed to the weaker ability of these mismatched targets to disassemble the CTGF HPs from nano-GO sheets. These results demonstrate the high specificity of the probe to CTGF target sequence.

Figure 3. Fluorescence spectra of the nano-GO/HP probe (10 $\mu\text{g/mL}$ nano-GO and 100 nM HP probe) in the presence of 200 nM target sequences with and without mismatched bases.

Cellular imaging of CTGF mRNA in abnormal fibroblasts

Prior to live cell imaging, we evaluated the stability of the nano-GO/HP probe against the nuclease. Both the probe and free MBs were treated with 2 U/mL endonuclease DNase I that can non-selectively degrade single- and double- stranded DNA. The degradation was monitored from the fluorescence restoration in the separation of dyes from quenchers (nano-GO sheets or BHQ2). As shown in [Figure S5](#), free CTGF MB exhibited a gradual fluorescence increasement and reached a plateau around 40 min after the DNase I treatment. However, there was no such phenomenon in the solution containing nano-GO/HP probe, which suggested that nuclease could not attack the HPs absorbed on the surface of nano-GO sheets.

Then, normal dermal fibroblasts (NDF) and fibroblasts derived from hypertrophic scars (HSF) were labeled with the probe. All the labeled cell groups exhibited red fluorescence 24 h post labeling, which demonstrated the successful visualization of intracellular CTGF mRNA. HSF exhibited much higher fluorescence signal compared with NDF, which was confirmed with both fluorescence imaging ([Figure 4a](#)) and flow cytometry ([Figure 4c](#)). This phenomenon was further confirmed by using RT-qPCR. For RT-qPCR analysis, the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal reference, and the fold change on the expression of CTGF mRNA relative to GAPDH mRNA was measured. As shown in [Figure 4b](#), HSF showed 8.2 times higher fold change of CTGF mRNA than NDF.

These results suggested that CTGF mRNA was highly expressed in HSF and is a suitable biomarker to allow abnormal scars to be clearly distinguished.

Figure 4. Identification of HSF from NDS with nano-GO/HP probe: (a) Representative fluorescence images of NDF and HSF unlabeled and labeled with the probe. Scale bar: 100 μ m. (b) Normalized probe fluorescence intensity (blue columns) from the labeled NDF and HSF measured by ImageJ, and fold change of CTGF mRNA relative to GAPDH mRNA (red columns) in NDF and HSF measured by RT-qPCR. ***, $p < 0.0001$; **, $p < 0.002$. (c) Flow cytometry analysis of NDF and HSF unlabeled and labeled with the probe.

Downregulation of transforming growth factor-beta type I receptor (TGF- β RI) expression by using TGF- β inhibitor Repsox or TGF- β RI siRNA can significantly suppress CTGF mRNA expression and reduce wound scarring.[26,16] Here we treated HSF with Repsox and TGF- β RI

siRNA. Compared to untreated HSF, the treated cell groups with either Repsox or TGF- β R1 siRNA exhibited much weaker red fluorescence signals (Figure 5a-b), which was confirmed by using flow cytometry analysis (Figure 5c). In addition, RT-qPCR analysis was also performed to evaluate the intracellular expression of CTGF mRNA relative to GAPDH mRNA (Figure 5b, red columns). After Repsox and TGF- β R1 siRNA treatment, the fold change of CTGF mRNA in HSF was decreased to 24.69 % and 16.41 % of that in the untreated HSF respectively, which was similar to the change of fluorescence intensity observed through the probe. These results demonstrated that the nano-GO/HP probe can achieve the noninvasive monitoring of CTGF mRNA expression during abnormal scar treatment with good reliability.

Figure 5. (a) Representative fluorescence images of HSF without and with the treatment of Repsox or TGF- β R1 siRNA before labeling with the nano-GO/HP probe (red channel) and Hoechst 33342 (blue channel). Scale bar: 100 μ m. (b) Normalized fluorescence intensity of CTGF-Cy3 signals (blue columns) from the untreated and treated HSF measured by ImageJ, and

fold change of CTGF mRNA relative to GAPDH mRNA (red columns) in HSF measured by RT-qPCR. ***, $p < 0.0005$; **, $p < 0.001$; *, $p < 0.005$. (c) Flow cytometry analysis of HSF untreated and treated with Repsox or TGF- β RI siRNA.

Discussion

This study demonstrated how a novel nano-GO probe detecting CTGF mRNA can differentiate between hypertrophic and normal dermal fibroblasts. This may allow it to detect abnormal scar cells at earlier stages of wound remodeling (prior to forming a mature scar lesion) and eventually monitor abnormal scar treatment. The nano-GO was employed as both the nanocarrier and the fluorescence quencher of CTGF HP probe. We assessed the intracellular CTGF mRNA expression through noninvasive fluorescence imaging based on the complexes of nano-GO and Cy3-labeled CTGF HP probe. The designed CTGF HP probe exhibited rapid response, high sensitivity and specificity to CTGF target sequence.

We note several benefits in the development of our nano-GO-based CTGF molecular probe. First, the conjugation of CTGF HP probe with nano-GO can shield the nucleic acid component (its recognition sequence) from degradation by intracellular nuclease. Second, confirmed by RT-qPCR analysis, much higher levels (8.2 times) of CTGF mRNA were observed in the abnormal scar fibroblasts, which confirms that higher CTGF mRNA expression can discriminate abnormal scar cells from undiseased cells. Third, the typical TGF- β antagonists Repsox and TGF- β RI siRNA can significantly silence the expression of CTGF mRNA in the hypertrophic scar fibroblasts, and this silencing can be evaluated by employing the nano-GO/CTGF HP probe. Overall, this paper demonstrated that the two-dimensional nano-GO probe can identify diseased scar cells from undiseased ones. It may eventually be applied to identify abnormal scars at an early stage.

Currently, nano-GO application in skin is limited by its size (in the order of several hundred nanometers). This inhibits the passive penetration of the nano-GO into skin because the nanoparticles with small particle size (< 10 nm) may passively penetrate the stratum corneum and reach the deeper layers of the skin.[27] In addition, the biocompatibility of the nano-GO may be another concern for its clinical application.[28,29] The functionalization of nano-GO with PEG or folic acid-conjugated chitosan oligosaccharide can address this issue.[21,30,31] Therefore, development of novel methods for preparing smaller sized nano-GO with appropriate

functionalization would promote the potential application of nano-GO in the skin area. Moreover, besides the CTGF mRNA detection in skin scar cells with and without drug treatment, this platform could also be easily re-developed for monitoring other *in vitro* interactions of drugs and skin cells by conjugating specific nucleic acid probes.

Conclusion

This article reports a CTGF mRNA sensor that is constructed by the noncovalent assembly of the fluorescent HP and nano-GO sheets. The assembly exhibited high sensitivity and specificity for *in vitro* detection of target sequence of CTGF mRNA with the limit of detection of 0.7 nM. This probe allows the detection of high expression of CTGF mRNA in HSFs, and reveals the change of the CTGF mRNA after treatment with drugs. We believe this probe is a great example to show the potential application of nano-GO in biomedical sensing.

Conflicts of Interest

No authors have any conflict of interest

Acknowledgements

This work was supported by A*STAR Biomedical Research Council (IAF-PP grant), Singapore Ministry of Education Tier-1 Academic Research Funds (RG 131/15), National Natural Science Foundation of China (21705012, 21605012), and Primary Research & Development Plan of Jiangsu Province (BE2016770), and the Fundamental Research Funds for the Central Universities (106112017CDJXFLX0014).

References

1. Yordanov YP, Shef A Hypertrophic Scars and Keloids – Contemporary Concepts and Treatment Options. *Acta Medica Bulgarica*. 2014; 41:57-74.
2. Darzi MA, Chowdri NA, Kaul SK et al. Evaluation of various methods of treating keloids and hypertrophic scars: a 10-year follow-up study. *Br J Plast Surg*. 1992; 45:374-9.
3. Mustoe TA, Cooter RD, Gold MH et al. International clinical recommendations on scar management. *Plast Reconstr Surg*. 2002; 110:560-71.
4. Yang L, Scott PG, Dodd C et al. Identification of fibrocytes in postburn hypertrophic scar. *Wound Repair Regen*. 2005; 13:398-404.

5. Iqbal SA, Sidgwick GP, Bayat A Identification of fibrocytes from mesenchymal stem cells in keloid tissue: a potential source of abnormal fibroblasts in keloid scarring. *Arch Dermatol Res.* 2012; 304:665-71.
6. Miura N, Kabashima H, Shimizu M et al. Clinical impact of serum transforming growth factor- α mRNA as a predictive biomarker for the prognosis of fulminant hepatitis. *Hepatol Int.* 2008; 2:213-21.
7. Zhou HX, Yang MX, Wang Y et al. Plasma LUNX mRNA, a non-invasive specific biomarker for diagnosis and prognostic prediction of non-small cell lung cancer. *Am J Cancer Res.* 2016; 6:452-8.
8. Brauburger K, Cressey T, Mühlberger E Nonradioactive Northern Blot Analysis to Detect Ebola Virus Minigenomic mRNA. *Methods Mol Biol.* 2017; 1628:143-59.
9. Li Y, Wang K, Chen L et al. Quantification of mRNA Levels Using Real-Time Polymerase Chain Reaction (PCR). *Methods Mol Biol.* 2016; 1406:73-9.
10. He L, Lu D-Q, Liang H et al. Fluorescence Resonance Energy Transfer-Based DNA Tetrahedron Nanotweezer for Highly Reliable Detection of Tumor-Related mRNA in Living Cells. *ACS Nano.* 2017; 11:4060-6.
11. Zhang R, Gao S, Wang Z et al. Multifunctional Molecular Beacon Micelles for Intracellular mRNA Imaging and Synergistic Therapy in Multidrug-Resistant Cancer Cells. *Adv Funct Mater.* 2017; 27:1701027.
12. Wang M, Hou X, Wiraja C et al. Smart Magnetic Nanosensors Synthesized through Layer-by-Layer Deposition of Molecular Beacons for Non-Invasive and Longitudinal Monitoring of Cellular mRNA. *ACS Appl Mater Interfaces.* 2016; 8:5877-86.
13. Penn JW, Grobelaar AO, Rolfe KJ The role of the TGF- β family in wound healing, burns and scarring: a review. *Int J Burns Trauma.* 2012; 2:18-28.
14. Sisco M, Kryger ZB, O'Shaughnessy KD et al. Antisense inhibition of connective tissue growth factor (CTGF/CCN2) mRNA limits hypertrophic scarring without affecting wound healing in vivo. *Wound Repair Regen.* 2008; 16:661-73.
15. Alfaro MP, Deskins DL, Wallus M et al. A physiological role for connective tissue growth factor in early wound healing. *Lab Invest.* 2013; 93:81-95.
16. Ide M, Jinnin M, Tomizawa Y et al. Transforming growth factor β -inhibitor Repsox downregulates collagen expression of scleroderma dermal fibroblasts and prevents bleomycin-induced mice skin fibrosis. *Exp Dermatol.* 2017; 26:1139-43.
17. Yeo DC, Wiraja C, Paller AS et al. Abnormal scar identification with spherical-nucleic-acid technology. *Nat Biomed Eng.* 2018; 2:227-38.
18. Wang Y, Li Z, Liu M et al. Multiple-targeted graphene-based nanocarrier for intracellular imaging of mRNAs. *Anal Chim Acta.* 2017; 983:1-8.
19. Fan J, Zhang X, Cheng Y et al. Increasing the sensitivity and selectivity of a GONS quenched probe for an mRNA assay assisted with duplex specific nuclease. *RSC Adv.* 2017; 7:35629-37.
20. Lu CH, Yang HH, Zhu CL et al. A graphene platform for sensing biomolecules. *Angew Chem.* 2009; 48:4785-7.
21. Chen TT, Tian X, Liu CL et al. Fluorescence Activation Imaging of Cytochrome c Released from Mitochondria Using Aptameric Nanosensor. *J Am Chem Soc.* 2015; 137:982-9.
22. Stobiecka M, Dworakowska B, Jakiela S et al. Sensing of survivin mRNA in malignant astrocytes using graphene oxide nanocarrier-supported oligonucleotide molecular beacons.

Sens Actuators B: Chem. 2016; 235:136-45.

23. Choi HY, Lee TJ, Yang GM et al. Efficient mRNA delivery with graphene oxide-polyethylenimine for generation of footprint-free human induced pluripotent stem cells. *J Control Release*. 2016; 235:222-35.
24. Wang J, Ge XM, Liu ZL et al. Heterogeneous Electrocatalyst with Molecular Cobalt Ions Serving as the Center of Active Sites. *J Am Chem Soc*. 2017; 139:1878-84.
25. Dai B, Fu L, Zou Z et al. Rational design of a binary metal alloy for chemical vapour deposition growth of uniform single-layer graphene. *Nat Commun*. 2011; 2:522.
26. Wang YW, Liou NH, Cherng JH et al. siRNA-Targeting Transforming Growth Factor- β Type I Receptor Reduces Wound Scarring and Extracellular Matrix Deposition of Scar Tissue. *J Invest Dermatol*. 2014; 134:2016-25.
27. Prow TW, Grice JE, Lin LL et al. Nanoparticles and microparticles for skin drug delivery. *Adv Drug Deliv Rev*. 2011; 63:470-91.
28. Wang Y, Li Z, Weber TJ et al. In situ live cell sensing of multiple nucleotides exploiting DNA/RNA aptamers and graphene oxide nanosheets. *Anal Chem*. 2013; 85:6775-82.
29. Ryoo SR, Lee J, Yeo J et al. Quantitative and Multiplexed MicroRNA Sensing in Living Cells Based on Peptide Nucleic Acid and Nano Graphene Oxide (PANGO). *ACS Nano*. 2013; 7:5882-91.
30. Yang X, Niu G, Cao X et al. The preparation of functionalized graphene oxide for targeted intracellular delivery of siRNA. *J Mater Chem*. 2012; 22:6649-54.
31. Cao X, Feng F, Wang Y et al. Folic acid-conjugated graphene oxide as a transporter of chemotherapeutic drug and siRNA for reversal of cancer drug resistance. *J Nanopart Res*. 2013; 15:1965.