Graphene Oxide and Fluorescent Aptamer Based Novel Biosensor for Detection of 25-hydroxyvitamin D_3

Ritika Gupta
National Agri-Food Biotechnology Institute (NABI)
Sunaina Kaul
National Agri-Food Biotechnology Institute (NABI)
Vishal Singh
National Agri-Food Biotechnology Institute (NABI)
Sandeep Kumar
Guru Jambheshwar University of Science and Technology
Nitin Kumar Singhal (nitin@nabi.res.in)
National Agri-Food Biotechnology Institute (NABI)

Research Article

Keywords: 25-Hydroxyvitamin D3, Aptamer, Graphene Oxide, Förster Resonance Energy Transfer, Biosensor

Posted Date: March 26th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-334003/v1

Version of Record: A version of this preprint was published at Scientific Reports on December 1st, 2021. See the published version at https://doi.org/10.1038/s41598-021-02837-4.

Abstract

For maintaining the healthy metabolic status, vitamin D is a beneficial metabolite stored majorly in its pre-activated form, 25-hydroxyvitamin D_3 ($25(OH)D_3$). Due to its important role in bone strengthening, the study was planned to quantify $25(OH)D_3$ levels in our blood. Quantification techniques for $25(OH)D_3$ are costly thus requiring a need for a low cost, and sensitive detection methods. In this work, an economic, and sensitive sensor for the detection of $25(OH)D_3$ was developed using aptamer and graphene oxide (GO). Aptamer is an oligonucleotide, sensitive towards its target, whereas, GO with 2D nanosheets provides excellent quenching surface. Aptamer labeled with fluorescein (5', 6-FAM) is adsorbed by π -π interaction on the GO sheets leading to quenching of the fluorescence due to Förster resonance energy transfer (FRET). However, in the presence of $25(OH)D_3$, a major portion of aptamer fluorescence remains unaltered, due to its association with $25(OH)D_3$. However, in the absence, aptamer fluorescence gets fully quenched. Fluorescence intensity quenching was monitored using fluorescence spectrophotometer and agarose gel based system. The limit of detection of $25(OH)D_3$ by this method was found to be 0.15 µg/mL. Therefore, this method could come up as a new sensing method in the field of vitamin D detection.

Introduction

Vitamin D (Vit D) is a major regulatory fat soluble secosteroid moiety governing the skeletal growth and calcium homeostasis. Cholecaciferol (Vit-D_3/$25(OH)D_3$) being the stable form of Vit-D is the measurable moiety in serum. Low levels of Vit-D have been associated with health conditions like Osteomalacia, Depression, Parkinson's disease, Autoimmune diseases and Cancer[1,2]. Thus, testing of Vit-D levels can be considered as an essential marker to estimate the quality of life of a population. Serum values lower than 75 nmol/L or 30 ng/ml are considered to be Vit-D deficient[3]. According to latest reports, Vit-D deficiency is found prevalent in developing and developed countries alike. With 5.9% population in US, 7.4% in Canada, 13% in Europe and > 20% population in countries like India, Pakistan and Afghanistan. 490 million people have been estimated to be Vit-D deficient in India[4]. Thus, the levels being on lower side, mass testing is required for the well-being of the population. Currently techniques used for the serum estimations are radioimmunoassay and chemiluminiscent immunoassay. Other techniques available are High Performance Liquid Chromatography (HPLC-UV) and liquid chromatography combined with Mass spectroscopy (LC-MS). Chromatographic techniques have an add on feature of separate detection for $25(OH)D_2$ and $25(OH)D_3$ [5]. Though the detection of $25(OH)D_3$ with the presently used methods is possible but have limitations of their own. Immunoassays having poor antibody specificity with issues of cross-reactivity while chromatography techniques involving costly instruments that require trained staff for operation[6,7]. Due to these cost limitations and high maintenance issues, status monitoring of $25(OH)D_3$ is hampered. Thus, there is a need to have point of care instrumentation for testing[8].

To overcome these issues, scientists have been successful in developing electrochemical detection systems. Immunosensor reported by Kaur *et al.*, provided a quick response time of 12 min and stability of

10 days. Bimetallic Au-Pt nanoparticles were synthesized that were later deposited on APTES modified electrode. Immobilization of Ab-25(OH)D_3 played the role of providing specificity and sensitivity to the electrode[9]. On similar grounds, Anusha *et al.*, developed a glassy carbon electrode surface for immobilization of Lanthanum nanoparticles-graphene quantum dots coupled with zeolitic imidazolate framework (ZIF-8). Sensitive detection of Vit-D in human serum and urine samples was made possible by this method[10]. Chauhan *et al.*, have been working on different procedures for Vit-D detection and have come up with electrochemical sensors using polyacrylonitrile nanofibers with incorporated magnetic nanoparticles and gadolinium oxide nanorods. They were able to obtain enhanced sensitivity of metal oxide nanorods as compared to nanofibers[11]. On the contrary, Lee *et al.*, developed a gold nanoparticle based platform for colorimetric quantification of Vit-D levels in serum samples on smartphone[12].

Recently, aptamers have successfully come up as a detection tool that is stable, cheap and reproducible[13]. They are oligonucleotide equivalent of antibodies having specificity towards particular metabolites [14]. They generally adopt secondary structures like stem, loop, G-quadruplex and hairpin that further form 3D conformations in presence of particular metabolites which is responsible for their binding affinity and specificity[15,16,17]. SELEX (systematic evolution of ligands by exponential enrichment) is the technique that allows the aptamers to segregate depending on their specificity towards their target moieties[18]. Forces enhancing the aptamer target binding involve non-covalent interactions including electrostatic interactions, hydrogen bonding, Van der Waal's interaction and shape complementarity[19]. In order to be used as sensors, aptamers can be labeled with fluorophores, gold nanoparticles and quantum dots that can be visualized on their respective binding [20,21,22].

Graphene oxide (GO), one atom thick sheet of oxidized graphite has been efficiently used as fluorescence quencher[23]. Due to its solubility, inertness and surface functionality, GO has been a part of many detection systems[24,25]. It acts as an excellent quencher of fluorescence by the process of FRET. FRET (Fluorescence Resonance Energy Transfer) is the phenomenon of energy exchange between molecules associated in < 10nm of range. Graphene oxide (GO), one atom thick sheet of oxidized graphite is an excellent quencher of fluorescence by the process of FRET. Fluorophore tagged to the hairpin aptamer loses its fluorescence when in contact with GO due to π stacking interactions between the nucleobases of ssDNA and π ring of GO. Utilizing the specificity benefits of FRET based assay, sufficient work has been published on detection of biomolecules such as thrombin [26], pfldh[20], adenosine[27], kanamycin [28]. There is a lack of Vit-D based detection systems utilizing the similar phenomenon. Considering the stability, specificity and low cost of aptamers we planned to prepare a FRET based assay for Vit-D detection with the help of above explained VDBA-14 aptamer.

In this study, a fluorescent tagged aptamer based detection system was developed for the identification of 25(OH)D_3. Hairpin loop aptamer, stacks on GO due to its π-π and hydrophobic weak interactions causing the associated quenching (Fig. 1). As already mentioned quenching occurs due to FRET phenomenon[29]. Conversely in the presence of 25(OH)D_3, aptamer having high affinity towards its metabolite aligns its conformation around it, leaving the vicinity of GO, increasing the distance between fluorophore and GO thus, producing fluorescence. 25(OH)D_3 detection utilizing the FRET[30] phenomenon and the gel based detection have not been studied so far, thus producing the novelty factor of the work. The above phenomenon was also validated by spectral changes and as well as agarose gel electrophoresis.

Results/discussions

Characterization of GO

Ultraviolet visible spectroscopy (Shimadzu, UV-2700) and Transmission electron microscopy (JEOL, JEM 2100, operating at 200 kV) were used for the characterization of prepared GO. In Fig. 2 (a) GO absorbs at 230nm which attribute to the π-π* transition of aromatic C-C bond and also represent π-π* plasmon peak. TEM was carried out to see the surface morphology which clearly demonstrates the single layer structure of GO and the same can be seen in Fig. 2 (b). thickness of which can be estimated by Atomic Force Microscopy (AFM) results (Fig. 2(c)). Thickness of the exfoliated GO sheets were estimated to be < 5nm by AFM (Bruker 8 multimode scanning microscope).

Optimization of Graphene oxide concentration for efficient quenching

Aptamer used in the study is β-hairpin loop ssDNA. Sequence and structure (Fig.S1) was given in supplementary information. GO has come up as an effective energy acceptor due to its unique electronic properties[28]. Thus, the strategy used for developing the assay involved fluorescence quenching of the FAM-aptamer in the presence of GO. FAM modified aptamer when present in vicinity of GO was able to get its fluorescence quenched due to the phenomenon of FRET. In the presence of Vit-D_3, affinity of aptamer towards Vit-D allowed it to majorly bind to the target molecule leaving few unbound aptamers to be adsorbed on GO sheets by π stacking interactions. This assay allowed enhanced fluorescence values with respect to increasing concentrations of Vit-D. The amount of quenching obtained was observed by allowing the FAM-aptamer to be mixed with GO followed by centrifugation. Supernatant was used for the spectral studies as shown in Fig. 3(a). Quenching of fluorophore was proportional with increasing concentration of GO. As in Fig. 3(b), we deduced that more than 60% quenching was observed when 50 µg/mL GO was used. Thus, 50 µg/mL was selected as the optimum concentration for the quenching studies. Fluorescence intensity was taken using absorbance at 495nm and emission at 520nm on Agilent Cary Eclipse Fluorescence Spectrophotometer. Qualitative analysis of fluorescence quenching can also be visualized on 3% agarose gel electrophoresis, image was taken on Azure Biosystems c600 (whole gel image given in Fig.S4(a)). The zeta potential taken on Malvern, Zetasizer, Nano-ZS shown in Fig. 3(c), decreased from − 38mV to -33mV when GO non-covalently interacted with FAM-apt. Thus, indicating capping of a few negative charges of GO by nucleobases of ssDNA FAM-aptamer. Figure 3(d) represent the time required for sufficient quenching of fluorescence in the presence of GO. Minimum 5 minutes were required for maximum quenching. Thermogravimetric analysis (TGA) was performed on PerkinElmer TGA 8000. As could be visualized from Fig S2(a), fall in weight% upto 100°C corresponded to loss in adsorbed water. Degradative step commencing at 165°C was indicative of loss of hydroxyl and epoxy functional groups. The second fall in weight from 400°C was indicative of remaining oxygen containing groups[31]. Difference of 2% weight loss was obtained in GO-Apt till temperature 100°C and from 200°C to 600°C. Functional groups characteristic for GO were analyzed through FTIR PerkinElmer UATR Two instrument. FTIR spectra clearly shows peaks at 1591.6 cm^{-1}, 1374.41cm^{-1} and 1075.99 cm^{-1} indicative of C = C, O-H and C-O-C respectively as can be observed from Fig S2(b) [32,33]. On addition of aptamer, significant changes were not observed due to weak π-π interactions between aptamer and GO. Fig. S2 (c) showed characteristic peak of GO at 230 nm that corresponded to π-π* transition of C = C bonds[33]. Peak of aptamer was seen at 260 nm. Slight shift in spectra could be observed in GO-Apt.

Condition optimization and 25-hydroxyvitamin D_3 detection

For pH optimization, aptamer and 25(OH)D_3 were added in different pH buffer (3.5, 5.5, 7.4, 8.3, and 9.2). As acidic pH caused charge neutralization on the GO surface, (H^+ charge abundance nullified the negative charges present on GO), the electrostatic forces between aptamer and GO were enhanced and caused complete quenching of the aptamer the π-π stacking forces between aptamer and GO were enhanced and caused complete quenching of the aptamer even in the presence of 25(OH)D_3 [34]. The interactions were so strong that even the presence of 25(OH)D_3 was insufficient to regain the fluorescence. As it can be seen from Fig S3(a), maximum fluorescence was detected in pH 7.4 and 8.3 reaction mixtures. At pH 9.2, as reaction mixture became alkaline, negative charge accumulation caused repulsion between aptamer and GO. Thus, causing fluorescence to maintain and very less quenching was observed. Similar results were also confirmed from agarose gel electrophoresis as shown in Fig. S3(a) and whole gel image Fig. S4(d).

To obtain the optimum visualization, effect of temperature on aptamer and 25(OH)D_3 (5µg/mL) binding was also studied. Aptamer and 25(OH)D_3 were incubated at different temperatures (4°C, 16°C, 25°C, 37°C and 50°C). It was concluded from Fig S3(b) that 37°C was appropriate for efficient aptamer and 25(OH)D_3 binding. Agarose gel showing fluorescent bands confirms the 37°C is the optimum temperature for the assay with complete gel image in Fig. S4(e).

Aptamer and different concentration of 25(OH)D_3 were allowed to bind for 45 mins with gentle mixing. After the incubation time was over, free aptamers got stacked on the GO surface. On centrifugation, GO bound aptamers settle in pellet while 25(OH)D_3 bound aptamers remained in the supernatant that was detected through spectral absorption and also validated by 3% agarose gel. In Fig. 4(a) fluorescent intensity spectrum shows that with the increase of in the 25(OH)D_3 fluorescence increases. The same is confirmed by the relative fluorescence measure where in the presence of 25(OH)D_3, fluorescence signal is observed. It is similar to the on/off kind of assay where the signal is on in the presence of the analyte and off in its absence. Minimum concentration of 25(OH)D_3 that was detected using this assay was 0.30 µg/mL with precision but can detect 0.15 µg/mL as confirmed by Fig. 4(b) and Fig S4 (b). We could also see the increase in FAM signal in the agarose gel which again proves the reliability of the assay. In order to compare the efficacy of the present study with already existing tools for 25(OH)D_3 detection, Table 1. is given.

Table 1
Comparison of various techniques available for detection of Vit D.

Analytical method	**Target**	**Limit of Detection**	**Ref.**
Radioimmunoassay	Vit-D	2.8ng/mL	38
Bimetallic nanoparticles on APTES modified glass electrode	Vit-D	0.0049ng/mL	9
HPLC	Vit-D_2 & Vit-D_3	5ng/mL	39
Aptasensor using Graphene Quantum Dot-Au hybrid nanoparticles	25(OH)D_3	0.28ng/mL	40
Electrochemical immunosensor	Vit-D_3	0.12ng/mL	11
Electrochemical sensor using Gadolinium oxide nanorods	Vit-D_3	0.10ng/mL	41
LC-MS/MS	25(OH)D_3	5-200ng/mL	42
FRET	25(OH)D_3	150ng/mL	Our work

Specificity check and real-time sample analysis in mice serum

Specificity reaction involved separate aliquots of aptamer to be allowed to bind to 25(OH)D_3 similar moieties such as 25(OH)D_2 and CDA in order to check for cross reactivity. Maximum fluorescence intensity was observed in 25(OH)D_3 as can be seen in Fig. 5 (a) and (b) as compared to the other compounds proving the specificity of the aptamer. Gel quantitative analysis also proves the specificity of the assay (Fig. 5(b) and Fig. 4(c)).

25(OH)D_3 was supposed to be detected in human serum but in order to prove the usefulness of the assay, we detected 25(OH)D_3 in mice serum. Mice sera was used to mimic the human serum. In 50% serum, fluorescence is higher than in water or buffer [35]. In this case, sufficient quenching was observed in absence of 25(OH)D_3 whereas in the presence of 25(OH)D_3 spiked serum sample, fluorescence intensity was increased when compared with no 25(OH)D_3 control as could be observed in Fig. 6 (a) and (b).

Materials And Methods

Materials

25-hydroxyvitaminD_3 (25(OH)D_3), 25-hydroxyvitaminD_2 (25(OH)D_2), Chenodeoxycholic acid (CDA), Tris, Sodium chloride, Graphite flakes from Sigma (www.sigmaaldrich.com), Agarose from Lonza (www.lonza.com). Aptamer specific to vitamin D was taken from literature[36] and synthesized with 6-carboxyfluorescein (FAM) modifications at its 5'end by IDT. 96 well black round bottom plate from Corning, Thermoscientific (www.thermoscientific.com). Mice serum was taken from animal house facility at National Agri-Food Biotechnology Institute, Mohali, India under the ethical no. NABI/2039/CPCSEA/IAEC/2019.

Synthesis and characterizations of Graphene Oxide (GO)

GO was prepared by modified Hummers' method [37]. Briefly, a 9:1 mixture of concentrated H_2SO_4/H_3PO_4 (360:40 mL) was added to a mixture of graphite flakes (3.0 g, 1 wt. equiv.) and $KMnO_4$ (18.0 g, 6 wt. equiv.). The reaction was then heated to 50°C and stirred for 12 h. The reaction was cooled to RT and poured onto ice (400 mL) with 30% H_2O_2 (3 mL). Then filtered through muslin cloth. The filtrate was centrifuged (5380g for 30mins), and supernatant decanted. The remaining solid material was then washed two times in succession with 200 mL of water, 200 mL, 30% HCl, and 200 mL, ethanol and after each wash, the mixture was filtered through muslin cloth with the filtrate being centrifuged (5380g for 30mins) and the supernatant decanted away. The remaining material after washing was coagulated with 200 mL of ether, and centrifuged at 5380g for 30mins to remove ether. The pellet obtained was dissolved in water and 0.1M NaOH was added to make homogenous suspension followed by dialysis using deionized water and lastly lyophilized overnight at room temperature to find the concentration of the obtained GO. Prepared graphene oxide was characterized by UV-Vis spectroscopy, TEM imaging and AFM analysis.

Optimization of Graphene Oxide concentration, temperature and pH for efficient quenching and regaining assays

Aptamer (stock 1µM) was prepared in 20 mM phosphate buffer, 25 mM NaCl, pH 7.4. To estimate the optimum concentration of GO, its varying concentrations (0, 12.5, 25, 50, 100 µg/ml) were incubated with aptamer, 100 nM (working concentration) for 10 minutes in 250 µl reaction mixture at room temperature.

Centrifugation was followed at 18800g for 20mins. Supernatant obtained was separated, used for fluorescence estimations and for visualized confirmation on agarose gel (3%).

To study the effects of pH on the binding, 5 μg/mL of 25(OH)D_3 was incubated with aptamer in varying reaction buffers, 20 mM sodium acetate buffer (pH-3.5, 5.5), 20 mM phosphate buffer (pH-7.4), 20 mM tris buffer (pH-8.3, 9.2). In order to find the optimum temperature, reaction mixture containing aptamer and 25(OH)D_3, was incubated at 4C, 16C, 25C, 37C and 50C. After the appropriate condition for standardization was completed over 45 minutes, GO (optimized as above, 50 μg/ml) was added to the reaction mixture followed by centrifugation and later estimations.

Fluorescence detection of Vitamin D_3

In a 250μL reaction mixture, aptamer (100 nM) and 25(OH)D_3 (0.15, 0.3, 0.6, 1.25, 2.5, 5, 10 and 20 μg/mL) were incubated in 20 mM phosphate buffer (25 mM NaCl, pH7.4) for 45 minutes with gentle mixing at 450 rpm in thermomixer (Eppendorf). GO (50 μg/mL) was added and allowed to mix for 10 mins at room temperature. In control experiment, only aptamer and GO were taken without 25(OH)D_3. Centrifugation was followed with supernatant removal without disturbing the pellet and analysis by fluorescence estimation and agarose gel based visualization.

Selectivity and real-time sample analysis

Closely resembling analytes, 25(OH)D_3, 25(OH)D_2 and CDA was used for the specificity check of the assay. 5 μg/mL of each biomolecule was diluted in 20 mM phosphate buffer, pH 7.4. Optimized concentrations of aptamer and GO was added and analyzed using the same procedure as given above for the detection of 25(OH)D_3.

In order to determine practical applications in human serum sample, mice serum was taken as a real-time sample. Serum was diluted to 50% with phosphate buffer, spiked up with 25(OH)D_3 in dilutions ranging from 0.15, 0.3, 0.6, 1.25, 2.5, 5,10 μg/mL were used for the estimations. For the control experiment, non-spiked sample of 50% mice serum was used. Rest of the assay procedure was followed as described above.

Conclusion

Vitamin D_3 being an essential component of the body requires proper detection system. As already shown that using aptamer based detection techniques, quantification becomes specific without any cross reactivity, which makes it a sensitive assay using fluorescence techniques. Here, for the first time FRET assay was used for fluorescent detection of 25(OH)D_3 and also for the visualization of the same on agarose gel. Increasing gradation of 25(OH)D_3 caused fluorescence restoration in phosphate buffer as well as similar results was observed in even 50% serum. Even the assay utilizing VDBA 14 aptamer has been reported to have LOD of 1μM which is far away from 0.006 μM as reported by Anusha et al., 2020[8]. Improvement in this protocol by either increasing the specificity of the aptamer and reducing the fluorescence constraints can help in development of chip model and used as point of care testing device. Limit of detection of this system is 0.15 μg/mL (1.5μM) which is roughly similar to the one obtained in literature. From the results, we can see that the quenching and fluorescence restoration could be visualized through optical gel imaging. Thus, making optical detection also possible. This technique could be used on chip model and can be used as point of care testing device. Further modifications could make the method more sensitive in the future.

Declarations

Conflict of interest

The authors declare that they have no competing interest.

Acknowledgment

The authors would like to thank Department of Biotechnology, New Delhi and National Agri-Food Biotechnology Institute, Mohali.

Author contributions

Ritika Gupta performed all the fluorescent spectroscopy experimental procedures and data collection. Sunaina Kaul performed gel-based experiments and prepared respective figures. Vishal Singh synthesized and characterized Graphene Oxide sheets. Sandeep Kumar helped in analyzing and interpreting the data. Nitin Kumar Singhal proposed the idea behind the study, done experimental planning, execution, data interpretation, drafting and critical editing of the article.

References

1. Wang, H. *et al.* Vitamin D and chronic diseases. *Aging Dis.* (2017) doi:10.14336/AD.2016.1021.
2. Holick, M. F. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. *The American journal of clinical nutrition* (2004).
3. Holick, M. F. Vitamin D Status: Measurement, Interpretation, and Clinical Application. *Annals of Epidemiology* (2009) doi:10.1016/j.annepidem.2007.12.001.
4. Amrein, K. *et al.* Vitamin D deficiency 2.0: an update on the current status worldwide. *European Journal of Clinical Nutrition* (2020) doi:10.1038/s41430-020-0558-y.
5. Altieri, B. *et al.* Vitamin D testing: advantages and limits of the current assays. *European Journal of Clinical Nutrition* (2020) doi:10.1038/s41430-019-0553-3.

6. Hutchinson, K., Healy, M., Crowley, V., Louw, M. & Rochev, Y. Verification of Abbott 25-OH-vitamin D assay on the architect system. *Pract. Lab. Med.* (2017) doi:10.1016/j.plabm.2017.01.001.
7. Lee, J. H., Choi, J.-H., Kweon, O. J. & Park, A. J. Discrepancy between Vitamin D Total Immunoassays due to Various Cross-reactivities. *J. Bone Metab.* (2015) doi:10.11005/jbm.2015.22.3.107.
8. Vemulapati, S., Rey, E., O'Dell, D., Mehta, S. & Erickson, D. A Quantitative Point-of-Need Assay for the Assessment of Vitamin D3 Deficiency. *Sci. Rep.* (2017) doi:10.1038/s41598-017-13044-5.
9. Kaur, A. *et al.* Gold-platinum bimetallic nanoparticles coated 3-(aminopropyl)triethoxysilane (APTES) based electrochemical immunosensor for vitamin D estimation. *J. Electroanal. Chem.* (2020) doi:10.1016/j.jelechem.2020.114400.
10. Anusha, T., Bhavani, K. S., Kumar, J. V. S. & Brahman, P. K. Designing and fabrication of electrochemical nanosensor employing fullerene-C60 and bimetallic nanoparticles composite film for the detection of vitamin D3 in blood samples. *Diam. Relat. Mater.* (2020) doi:10.1016/j.diamond.2020.107761.
11. Chauhan, D., Gupta, P. K. & Solanki, P. R. Electrochemical immunosensor based on magnetite nanoparticles incorporated electrospun polyacrylonitrile nanofibers for Vitamin-D3 detection. *Mater. Sci. Eng. C* (2018) doi:10.1016/j.msec.2018.07.036.
12. Lee, B. H., Nguyen, V. T. & Gu, M. B. Highly sensitive detection of 25-HydroxyvitaminD3 by using a target-induced displacement of aptamer. *Biosens. Bioelectron.* (2017) doi:10.1016/j.bios.2016.08.011.
13. Zichel, R., Chearwae, W., Pandey, G. S., Golding, B. & Sauna, Z. E. Aptamers as a sensitive tool to detect subtle modifications in therapeutic proteins. *PLoS One* (2012) doi:10.1371/journal.pone.0031948.
14. Zhou, J. & Rossi, J. Aptamers as targeted therapeutics: Current potential and challenges. *Nature Reviews Drug Discovery* vol. 16 181–202 (2017).
15. Sullivan, R., Adams, M. C., Naik, R. R. & Milam, V. T. Analyzing secondary structure patterns in DNA aptamers identified via compels. *Molecules* (2019) doi:10.3390/molecules24081572.
16. Liu, C. *et al.* Target-induced structure switching of a hairpin aptamer for the fluorescence detection of zeatin. *Anal. Methods* (2016) doi:10.1039/c6ay01679k.
17. Wang, Y. *et al.* A novel aptamer-quantum dot fluorescence probe for specific detection of antibiotic residues in milk. *Anal. Methods* (2016) doi:10.1039/c6ay00412a.
18. Zhuo, Z. *et al.* Recent advances in SELEX technology and aptamer applications in biomedicine. *International Journal of Molecular Sciences* vol. 18 (2017).
19. Cai, S. *et al.* Investigations on the interface of nucleic acid aptamers and binding targets. *Analyst* (2018) doi:10.1039/c8an01467a.
20. Godonoga, M. *et al.* A DNA aptamer recognising a malaria protein biomarker can function as part of a DNA origami assembly. *Sci. Rep.* **6**, 1–12 (2016).

21. Oh, S. Y. *et al.* Development of gold nanoparticle-aptamer-based LSPR sensing chips for the rapid detection of Salmonella typhimurium in pork meat. *Sci. Rep.* (2017) doi:10.1038/s41598-017-10188-2.
22. Motaghi, H., Mehrgardi, M. A. & Bouvet, P. Carbon Dots-AS1411 Aptamer Nanoconjugate for Ultrasensitive Spectrofluorometric Detection of Cancer Cells. *Sci. Rep.* (2017) doi:10.1038/s41598-017-11087-2.
23. Liu, Y., Liu, C. Y. & Liu, Y. Investigation on fluorescence quenching of dyes by graphite oxide and graphene. *Appl. Surf. Sci.* (2011) doi:10.1016/j.apsusc.2010.12.136.
24. Geim, A. K. & Novoselov, K. S. Rise of Graphene Nature Materials 2007 6 191. *Nat. Mater.* **6**, 183–191 (2010).
25. Wang, H. *et al.* Graphene oxide-peptide conjugate as an intracellular protease sensor for caspase-3 activation imaging in live cells. *Angew. Chemie - Int. Ed.* (2011) doi:10.1002/anie.201101351.
26. Altschuler, S. E., Dickey, T. H. & Wuttke, D. S. Schizosaccharomyces pombe Protection of Telomeres 1 Utilizes Alternate Binding Modes to Accommodate Different Telomeric Sequences. *Biochemistry* **50**, 7503–7513 (2011).
27. Bai, Y. *et al.* A turn-on fluorescent aptasensor for adenosine detection based on split aptamers and graphene oxide. *Analyst* **139**, 1843–1846 (2014).
28. Ha, N. R., Jung, I. P., La, I. J., Jung, H. S. & Yoon, M. Y. Ultra-sensitive detection of kanamycin for food safety using a reduced graphene oxide-based fluorescent aptasensor. *Sci. Rep.* **7**, 1–10 (2017).
29. Wang, Y. *et al.* Aptamer/graphene oxide nanocomplex for in situ molecular probing in living cells. *J. Am. Chem. Soc.* (2010) doi:10.1021/ja103169v.
30. Huang, A., Li, W., Shi, S. & Yao, T. Quantitative Fluorescence Quenching on Antibody-conjugated Graphene Oxide as a Platform for Protein Sensing. *Sci. Rep.* (2017) doi:10.1038/srep40772.
31. Chen, W., Yan, L. & Bangal, P. R. Preparation of graphene by the rapid and mild thermal reduction of graphene oxide induced by microwaves. *Carbon N. Y.* (2010) doi:10.1016/j.carbon.2009.11.037.
32. Kuila, T. *et al.* A green approach for the reduction of graphene oxide by wild carrot root. *Carbon N. Y.* **50**, 914–921 (2012).
33. Chen, J., Li, Y., Huang, L., Li, C. & Shi, G. High-yield preparation of graphene oxide from small graphite flakes via an improved Hummers method with a simple purification process. *Carbon N. Y.* **81**, 826–834 (2015).
34. Wu, M., Kempaiah, R., Huang, P. J. J., Maheshwari, V. & Liu, J. Adsorption and desorption of DNA on graphene oxide studied by fluorescently labeled oligonucleotides. *Langmuir* **27**, 2731–2738 (2011).
35. Bruno, J. G., Carrillo, M. P., Phillips, T. & Edge, A. Serum inverts and improves the fluorescence response of an aptamer beacon to various vitamin D analytes. *Luminescence* (2012) doi:10.1002/bio.1324.
36. Lee, B. H., Nguyen, V. T. & Gu, M. B. Highly sensitive detection of 25-HydroxyvitaminD3 by using a target-induced displacement of aptamer. *Biosens. Bioelectron.* **88**, 174–180 (2017).

37. Marcano, D. C. *et al.* Improved synthesis of graphene oxide. *ACS Nano* (2010) doi:10.1021/nn1006368.

38. Hollis, B. W., Kamerud, J. Q., Selvaag, S. R., Lorenz, J. D. & Napoli, J. L. Determination of vitamin D status by radioimmunoassay with an 125I- labeled tracer. *Clin. Chem.* (1993) doi:10.1093/clinchem/39.3.529.

39. Lensmeyer, G. L., Wiebe, D. A., Binkley, N. & Drezner, M. K. HPLC method for 25-hydroxyvitamin D measurement: Comparison with contemporary assays. *Clin. Chem.* (2006) doi:10.1373/clinchem.2005.064956.

40. Wadhwa, S., John, A. T., Nagabooshanam, S., Mathur, A. & Narang, J. Graphene quantum dot-gold hybrid nanoparticles integrated aptasensor for ultra-sensitive detection of vitamin D3 towards point-of-care application. *Appl. Surf. Sci.* (2020) doi:10.1016/j.apsusc.2020.146427.

41. Chauhan, D., Kumar, R., Panda, A. K. & Solanki, P. R. An efficient electrochemical biosensor for Vitamin-D3 detection based on aspartic acid functionalized gadolinium oxide nanorods. *J. Mater. Res. Technol.* (2019) doi:10.1016/j.jmrt.2019.09.017.

42. F Ozbakir, H. & A Sambade, D. Detection of 25-Hydroxyvitamin D3 with an Enzyme modified Electrode. *J. Biosens. Bioelectron.* (2016) doi:10.4172/2155-6210.1000193.

Figures

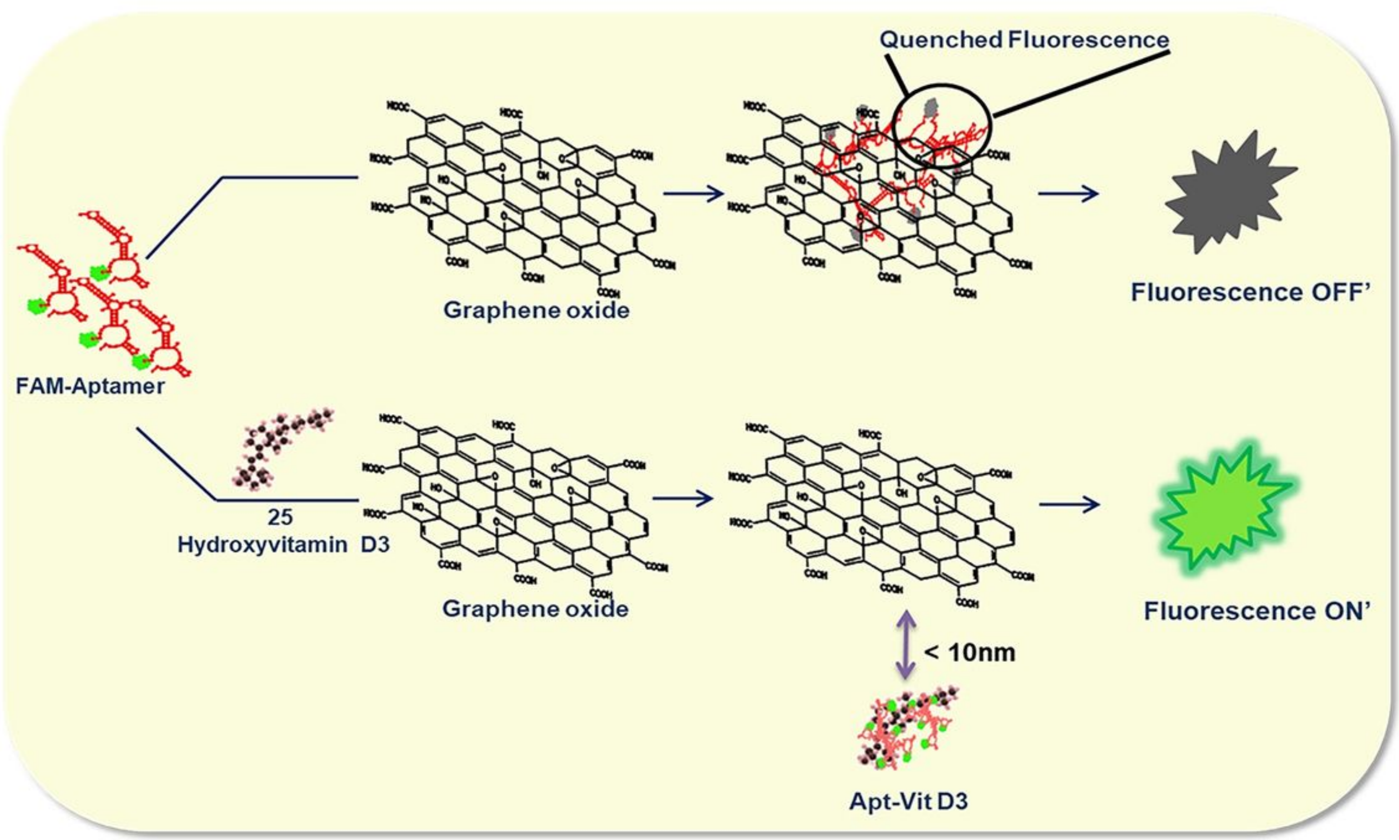

Figure 1

Schematic representation of FAM-Aptamer. Fluorescence is quenched by GO in absence of 25(OH)D3 while in its presence, gain of fluorescence is observed due to aptamer-25(OH)D3 binding.

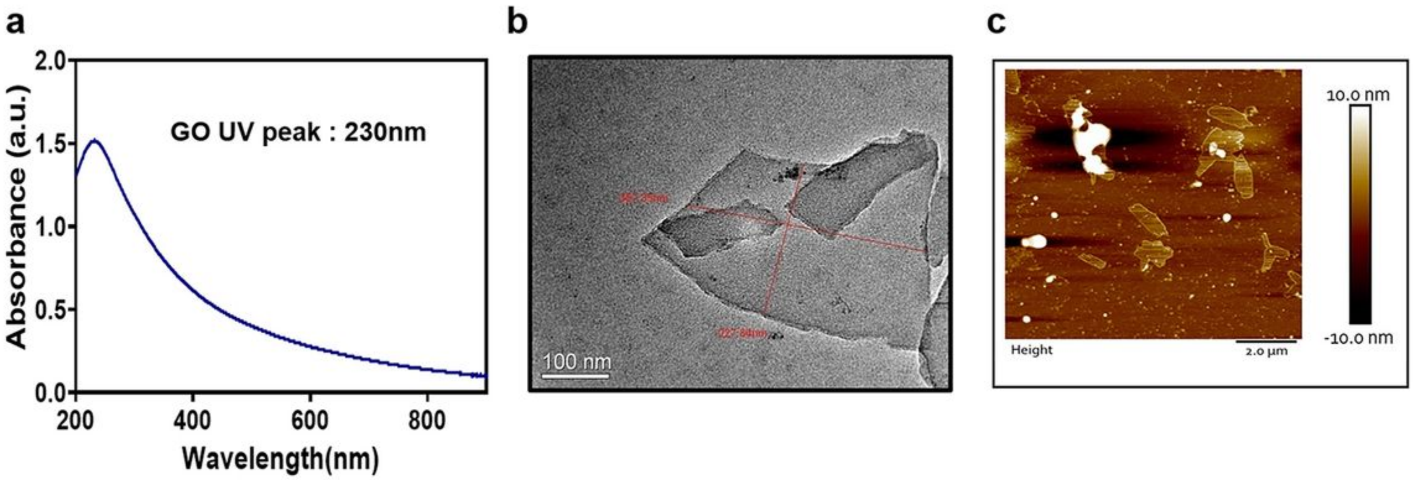

Figure 2

Characterizations of synthesized Graphene Oxide (a) UV spectra of GO (b) TEM image of GO (c) AFM image of GO

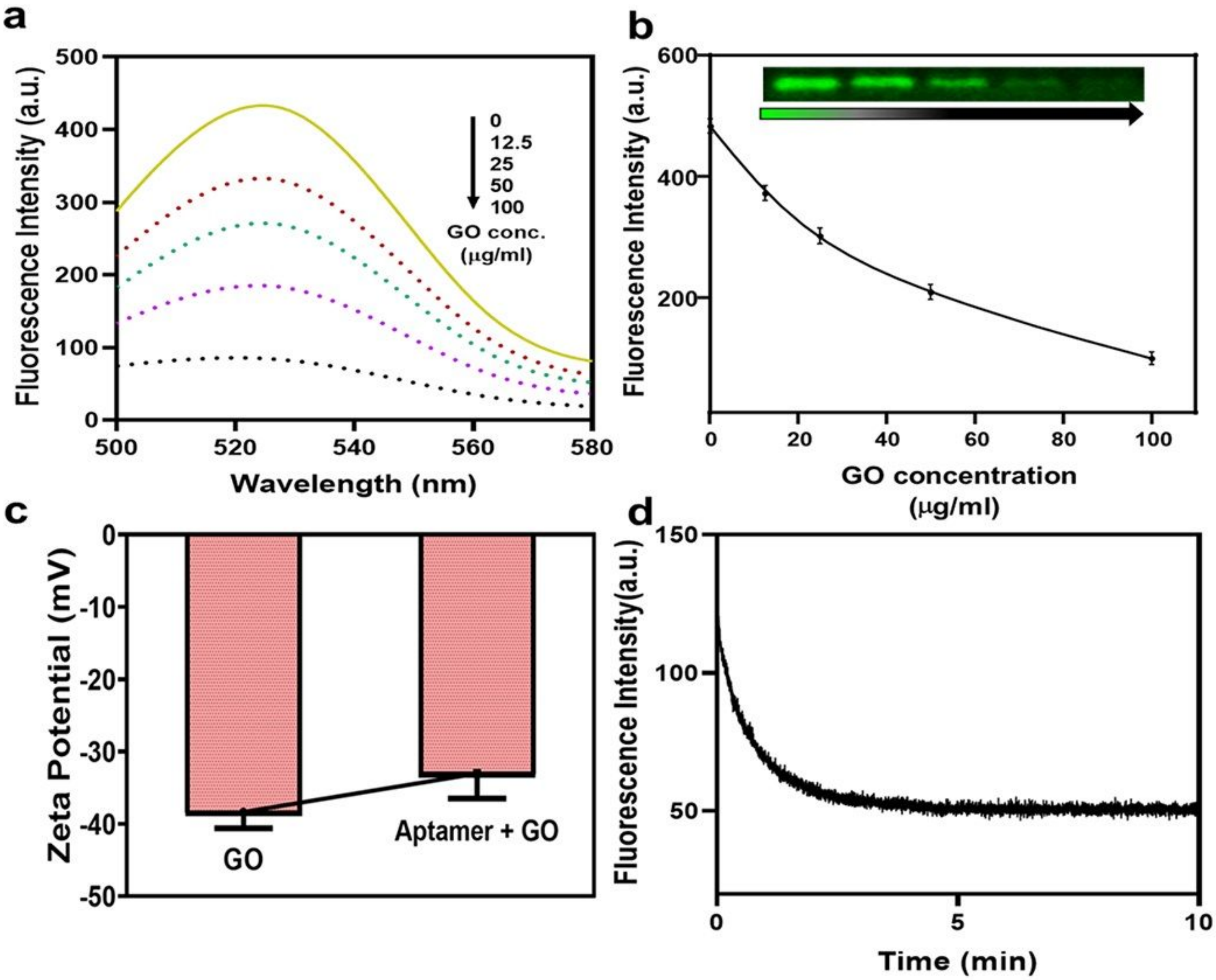

Figure 3

Graphene oxide and aptamer optimization (a) Fluorescent spectra of aptamer with various concentration of GO. (12.5 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL) (b) Quantitative (agarose gel) and qualitative results of aptamer fluorescence with increasing concentration of GO. Inset: Peak fluorescence change shows a linear relationship with Graphene oxide concentration. (c) Zeta potential of Apt and Apt-GO (d) Time kinetics of fluorescence quenching in presence of 50 μg/mL

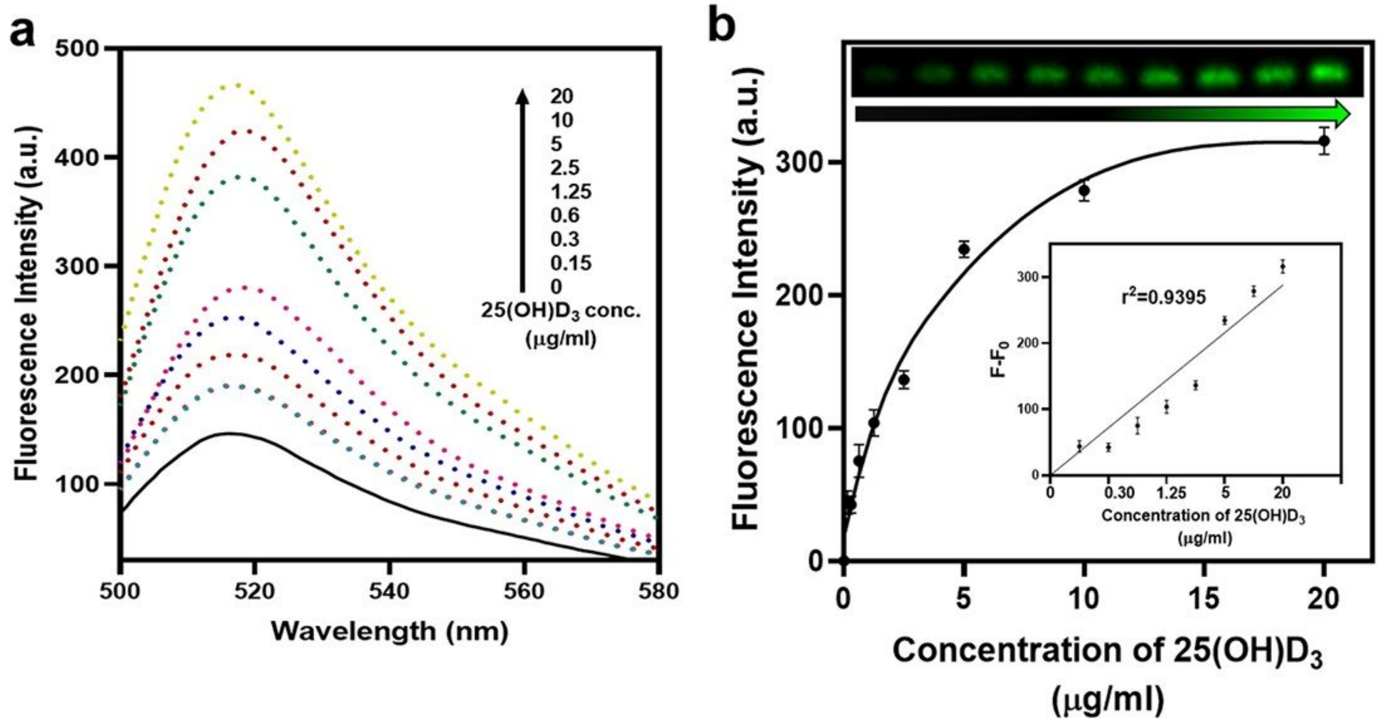

Figure 4

Assessment of fluorescence intensity obtained with 25(OH)D3 (a) Fluorescent spectra of the aptamer with different conc. of 25(OH)D3 (b) Fluorescent intensity of the aptamer with different concentrations of 25(OH)D3 and 3% agarose gel image confirming the increase in fluorescence in presence of 25(OH)D3. Inset: Peak fluorescence change shows a linear relationship with Graphene oxide concentration. (Apt – 100nM, GO-50μg/mL)

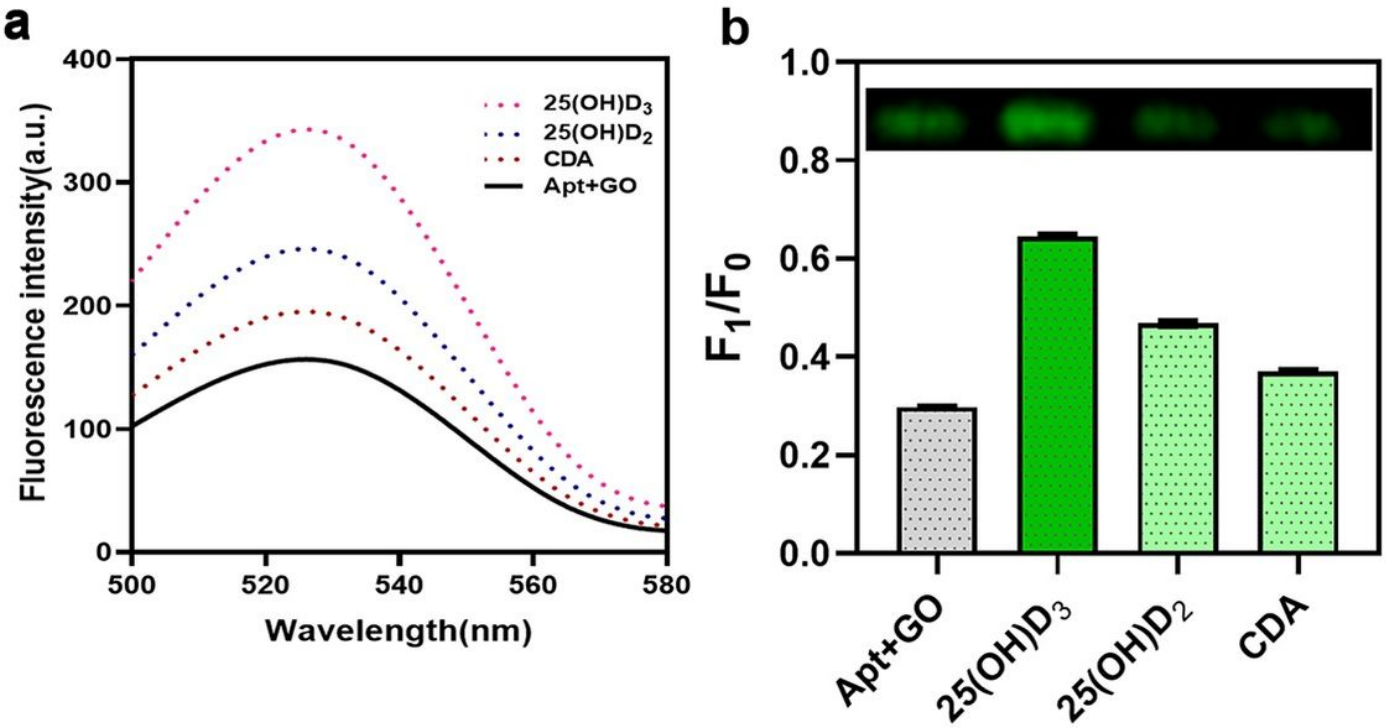

Figure 5

Specificity evaluation for 25(OH)D3 detection (a) Aptamer binding specificity check with different negative controls 25(OH)D2 and CDA. Maximum fluorescence intensity was observed in 25(OH)D3 when compared to the other compounds proving the specificity of the aptamer. (b) Quantitative and qualitative analysis was also proved using 3% agarose gel electrophoresis. [Apt – 100nM, GO-50μg/mL and 25(OH)D3- 5 μg/mL]

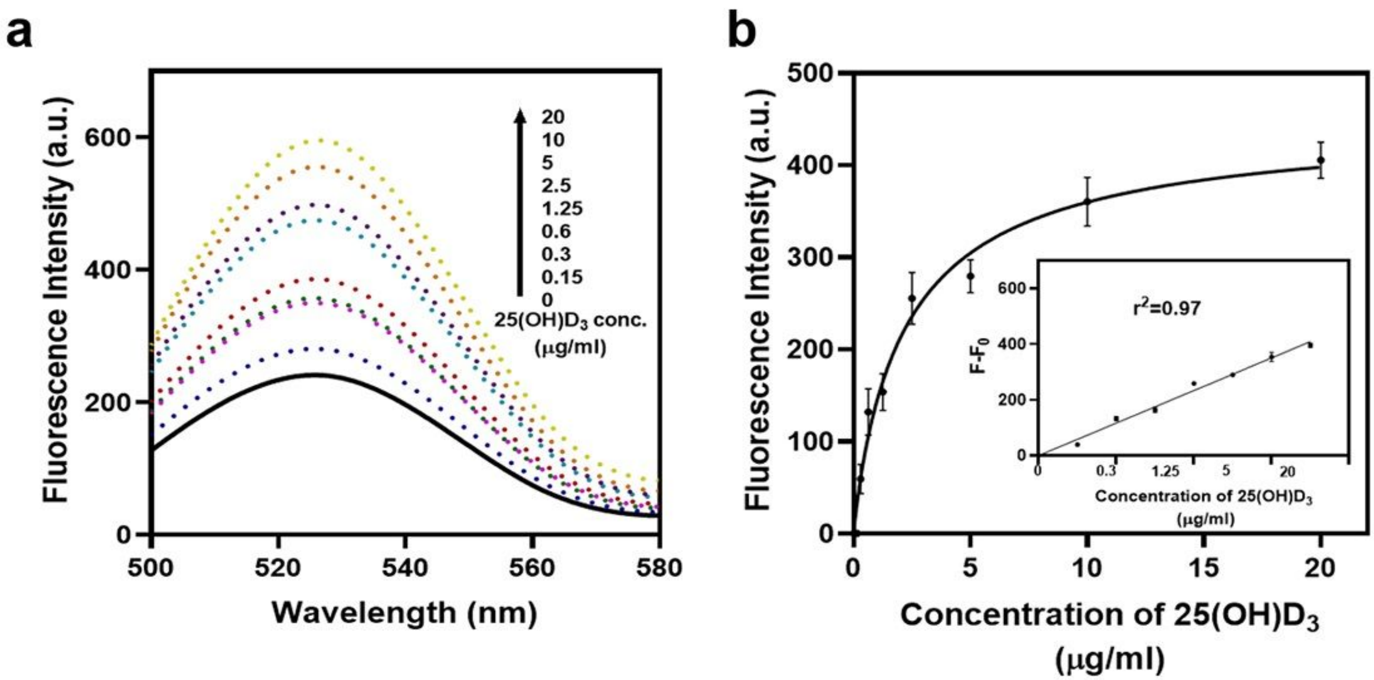

Figure 6

Evaluation for 25(OH)D3 detection in Mice serum. (a) Fluorescent spectrum of real time samples analysis using 50% Mice serum spiked up samples with 25(OH)D3 (b) Fluorescent intensity of the aptamer with different concentrations of 25(OH)D3 and 3% agarose gel image confirming the increase in fluorescence in presence of 25(OH)D3. Inset: Peak fluorescence change shows a linear relationship with Graphene oxide concentration. (Apt –100 nM, GO-50 μg/mL).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryinformationNSR.pdf