RESEARCH PAPER

Nanozyme-based Aptasensor for colorimetric detection of vitamin D_3 in milk

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ABSTRACT

Objective(s): The growing demand for food production necessitates the development of advanced detection methods to ensure food quality and prevent adulteration. This study presents a colorimetric aptasensor specifically designed to detect vitamin D3 in milk, utilizing the unique properties of nanozymes in combination with aptamers.

Materials and Methods: The method utilizes the peroxidase-like activity of bare iron oxide magnetic nanoparticles, which interact electrostatically with aptamers, leading to a color change in the TMB-H2O2 solution through a Fenton-like redox reaction.

Results: The results demonstrated that both the choice of buffer and the concentration of vitamin D3 significantly impact the catalytic activity of the nanozymes. In addition, using iron oxide nanozymes offers several advantages, including enhanced stability, straightforward interactions, tunable activity, and effective background color removal through magnetic separation.

Conclusions: This innovative approach enhances the reliability of vitamin D3 detection in dairy products and holds broader implications for food safety and quality assurance. The findings highlight the potential of integrating nanotechnology and biosensing techniques to address critical challenges in food monitoring and safety. By tackling these issues, this research contributes to the development of effective strategies for ensuring food integrity and safeguarding consumer health in an era where food adulteration continues to be a pressing concern.

Keywords: *Iron oxide nanoparticles; Vitamin D_3; Peroxidase; Oligonucleotides; Colorimetry; Milk*

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INTRODUCTION

Dairy products have long been a cornerstone of the human diet, providing essential nutrients and energy in various forms. These products' core is milk, the primary ingredient in a wide range of dairy offerings. The dairy industry faces the critical responsibility of ensuring milk quality, especially since processing can alter its composition, leading to reductions in fat content and vital nutrients such as vitamin D3 [1,2]. To address these changes, fortified dairy products—particularly vitamin D3-fortified milk—have emerged as a solution to replenish lost nutrients and meet consumer demand. This trend has increased production and raised concerns regarding profit-driven practices and the potential for adulteration within the industry. As a result, there is an urgent need for innovative technologies to effectively monitor and prevent dairy adulteration.

Vitamin D3, or cholecalciferol, is in relatively low concentrations in milk and other foods, complicating monitoring efforts. Advanced equipment and technologies are essential for detecting and preventing adulteration [4,5]. Previous research has explored various methods to measure vitamin D3 levels in milk and other foods, including chromatography, spectrophotometry, antibody-based methods, and aptamer-based assays [6-9]. Among these, High-Performance Liquid Chromatography (HPLC) is widely recognized as the standard for quantifying cholecalciferol in milk. However, it presents challenges related to quality control and the monitoring of fortified products. These challenges include complex sample preparation, high maintenance costs for equipment, reliance on skilled operators, and time-consuming processes for analyzing multiple samples [10-12].

In recent decades, aptamer-based detection methods have gained significant attention as

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advanced diagnostic platforms. Aptamers are short, single-stranded oligonucleotides (less than 25 kDa) capable of binding to specific targets with high affinity through various interactions [13,14]. This unique property enables the design of diverse aptasensors. One prominent approach involves colorimetric methods that utilize aptamers. Several variations of aptamer-based colorimetric methods have been developed, including those that employ enzyme-conjugated aptamers, G-quadruplex aptamers, hemin-based systems, and aptamer-gold nanoparticle complexes [15-20].

"Nanoparticles play a crucial role in enhancing the performance of aptamer-based diagnostics. Among these, iron oxide magnetic nanoparticles (MNPs) are particularly noteworthy due to their superparamagnetic properties, which facilitate the separation and stabilization of target analytes during diagnostic processes [21,22]. MNPs can also reduce background interference in colorimetric detection methods by eliminating background color from samples, which is an essential consideration as such interference can significantly affect assay outcomes. Additionally, MNPs exhibit enzyme-like behavior that generates signals in colorimetric assays [24-26]. Known as 'nanozymes,' these nanoparticles mimic enzymatic activity, with MNPs being particularly well-documented for their peroxidase-like activity.

"The interaction between aptamers and nanoparticles is fundamental to developing effective diagnostic methods. The electrostatic attraction between negatively charged aptamers and positively charged iron oxide nanoparticles facilitates their binding. This interaction modulates the enzyme-mimicking activity of MNPs: coating the nanoparticle surface with an aptamer inhibits its reaction with hydrogen peroxide (H2O2), thereby reducing peroxidase activity [33-35]. By forming an aptamer-nanozyme complex, the extent of peroxidase activity becomes directly proportional to the quantity of aptamer bound to the nanoparticle surface. This principle forms the basis for the design of colorimetric diagnostic methods utilizing iron oxide nanozymes and aptamers [33,36].

In addition to traditional approaches, recent advancements have highlighted innovative platforms for monitoring vitamin D levels. For instance, ferrocene-tagged primary antibodies have been employed to generate electrochemical signals for assessing vitamin D deficiency in clinical samples [37]. At the same time, disposable impedimetric nano-immunochips offer rapid diagnostics for vitamin D status [38]. Other notable developments include electrochemical immunosensors based on nanocomposites for vitamin D monitoring [39], novel lanthanum nanoparticles coupled with graphene quantum dots for sensing applications [40], handmade paper sensors utilizing silver-cobalt-doped copolymers [41], and an AuNP-based electrochemical aptasensor designed explicitly for detecting 25-hydroxy vitamin D3 [42]. These advancements underscore the dynamic landscape of diagnostic technologies that effectively address vitamin D deficiency. In summary, integrating advanced detection methods using aptamers and nanoparticles presents promising avenues for enhancing the quality control of fortified dairy products and ensuring consumer safety against adulteration.

In this study, we developed a detection assay based on a specific aptamer and magnetic nanozymes to detect milk cholecalciferol (vitamin D3). The specific aptamer against cholecalciferol was utilized for detection and as a surface coating agent for the nanoparticles, modulating the peroxidase reaction (Fig. 1).

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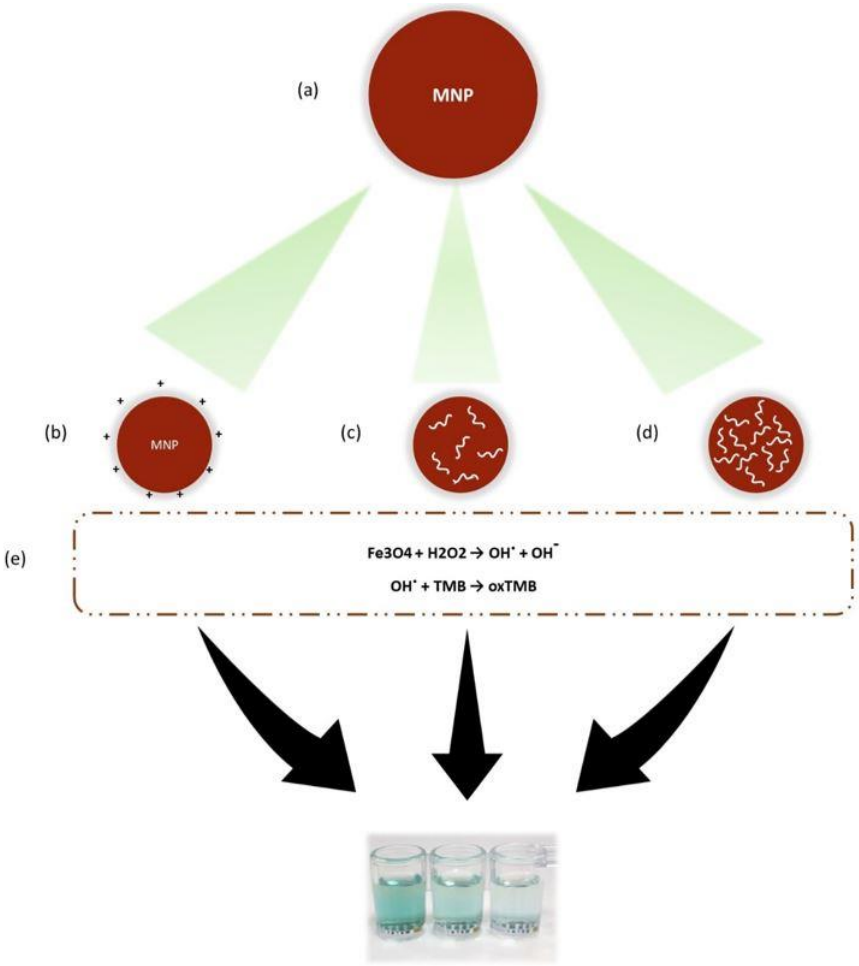

Fig 1. Investigating the effect of coating the surface of magnetic nanoparticles (MNPs) using aptamer: a) MNPs without coating and surface charge; b) MNPs suspended in a suitable buffer exhibiting a cationic surface charge; c) MNPs with surfaces partially coated by aptamers through electrostatic interactions; d) MNPs with a greater surface area covered by aptamers at a higher concentration; e) The reaction that describes the peroxidation and oxidation of TMB-H_2O_2, resulting in the production of a colored signal. The intensity of the color signal increases with the coating of MNPs by aptamers and an increase in their concentration, compared to uncoated MNPs.

MATERIALS AND METHODS

Chemicals and Instruments

The cholecalciferol-specific single-stranded oligonucleotide as the aptamer (5'-AGCAGCACAGAGGTCATGGGGGGTGTGACTTTGGTG TGCCTATGCGTGCTACGGAA-3') was selected based on previous reports [43, 44] and ordered for synthesis with HPLC-grade purification from Metabion International AG Co., Ltd. (Germany). The oligonucleotide powder was dissolved in double-distilled, deionized, sterile water before use. Cationic magnetite nanoparticles (Fe_3O_4 NPs) were purchased from Chemicell Co., Ltd. (Germany). 3,3',5,5'-tetramethylbenzidine (TMB) powder and all buffer salts were obtained from Sigma-Aldrich Co., LLC. (Milwaukee, WI, USA). Sodium acetate, acetic acid, and hydrogen peroxide (30%) were purchased from Asia Pajohesh (Iran). The stop reagent (sulfuric acid) was obtained from Pishtaz Teb Zaman (Iran). Vitamin D3 ampoules (30,000 IU) were obtained from Caspian Tamin Co., Ltd. (Iran). All reagents were of analytical grade, and bi-distilled water was used throughout the experiments. A Microreader (DANA-3200, Iran) and a Nanodrop UV-vis spectrophotometer (WPA Bio Wave II) were also used in this study.

Characterization of the aptamer-vitamin D3 interaction

The specific interaction between the aptamer and cholecalciferol forms the basis for developing the detection method. The possibility of this interaction was investigated using the spectrophotometric technique. This study focuses on changes in the UV absorption peak at 260 nm for DNA oligonucleotides (i.e., oligoaptamers). Mixtures with a constant concentration of 0.2 µM aptamer and varying concentrations of cholecalciferol (0.75

ppb, 0.075 ppb, 0.0075 ppb, 0.00075 ppb, and 0.000075 ppb) were prepared. Their UV spectra were recorded from 200 to 400 nm. The results were compared with control samples containing Vitamin D3 without the specific aptamer and mixtures containing the specific aptamer without Vitamin D3.

Fenton-like activity of MNPs in different buffers

Some studies have demonstrated that nanoparticles can exhibit diverse enzyme-mimicking behaviors under various conditions, with the reaction buffer being a crucial factor influencing these behaviors [45]. Specifically, two commonly used buffers in related studies are the acetate buffer (pH 4) and the binding buffer (Tris 20 mM, $CaCl_2$ 1 mM, KCl 5 mM, $MgCl_2$ 2 mM, NaCl 100 mM). This study investigated the peroxidase-like activity of MNPs in different buffers. Solutions containing a constant concentration of 6.25 µg/ml nanoparticles were prepared in both buffers. Then, 50 µL of TMB-H_2O_2 was added to the microwells, and after 30 minutes, a stopping solution was introduced. The optical density of the wells at 450 nm was subsequently evaluated.

Peroxidase activity of aptamer-assisted MNPs

Peroxidase activity is mediated by nanozymes on the surface of nanoparticles. Previous studies have shown that MNPs exhibit an oxidation-reduction reaction in the presence of H_2O_2, known as the Fenton-like reaction, which can occur on the surface of bare MNPs. To investigate the peroxidase activity of aptamer-assisted MNPs, different concentrations (0.25 µM and 0.5 µM) of aptamer were incubated for 15 minutes in a 0.2 M acetate buffer with a constant concentration of 6.25 µg/ml of MNPs. Then, 50 µL of TMB-H_2O_2 was added to the wells. After 30 minutes, a stopping solution was introduced. The optical density of the wells at 450 nm was then evaluated.

Optimization of the ratio of aptamers to MNPs

Since the interaction between the aptamer and MNPs alters nanozyme activity, optimizing the aptamer concentration relative to the nanoparticle concentration is crucial. Additionally, the detection mixture should exhibit minimal background color from the nanoparticles to reduce interference with the color signal. To achieve this, 6.25 µg/mL of MNPs were incubated with different aptamer concentrations (5 nM) in a 0.2 M acetate buffer for 15 minutes. Subsequently, 50 µL of TMB-H_2O_2 was added to the wells. After a 30-minute incubation, the stopping solution was introduced. The optical density of the wells was then measured at a wavelength of 450 nm.

Limit of detection of the nanozyme-assisted aptamers for cholecalciferol

The diagnostic method is based on the competition between the binding of an aptamer to Vitamin D3. To determine the optimal concentration of MNPs (6.25 µg/mL), various concentrations of cholecalciferol (375 ng/mL, 37.5 ng/mL, 3.75 ng/mL, 0.375 ng/mL, and 0.0375 ng/mL) were mixed with 5 nM of aptamer. A 15-minute incubation period was used for the detection process. Subsequently, 50 µL of TMB-H_2O_2 was added to each well. After 30 minutes, a stopping solution was introduced. The optical density was then measured at a wavelength of 450 nm.

Detection of vitamin D_3 in milk samples

Different concentrations of cholecalciferol were prepared in the milk to detect cholecalciferol in milk samples. The colorimetric method was performed in two modes: without removing the milk background color and with the background color removed. The optimized values were used to evaluate the detection method without removing the background color. Milk samples prepared with different concentrations of Vitamin D3 were first centrifuged at 20,000 ×g for 10 minutes at 25°C. The supernatant was then collected and added to the detection mixture. In the second mode, the sample background color was removed using the superparamagnetic properties of MNPs. After 15 minutes of Vitamin D3 recognition by the aptamer for magnetic separation, the reaction mixtures were exposed to an external magnetic field for 10 minutes. The MNPs were then accumulated, and the supernatant was discarded. A 0.2 M acetate buffer was added, and the washing process was repeated twice. Finally, according to the optimized protocol, the chromogen-substrate solution was added. After stopping the color changes with the stopping solution, the optical density of the wells at 450 nm was measured.

RESULTS AND DISCUSSION

Characterization of aptamer-vitamin D3 interactions

The detection of cholecalciferol using a specific aptamer relies on a stable interaction between the aptamer and Vitamin D3. Spectroscopic analysis revealed that the aptamer-Vitamin D3 interaction changed the optical absorption spectrum, forming a new complex with a spatial structure distinct from the initial aptamer.

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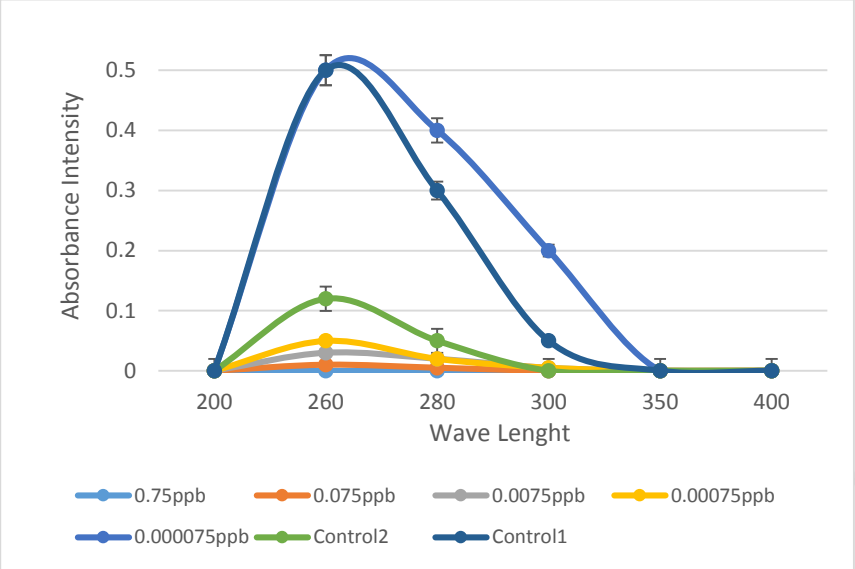

Fig 2. The absorption spectrum of the interaction 0.2 µM of aptamer and different concentrations of cholecalciferol (0.000075 ppb, 0.00075 ppb, 0.0075 pbb, 0.075 ppb, 0.75 pbb); Control 1: including 0.2 µM aptamer without Vit D3; Control 2: including 0.75ppb Vit D3 without aptamer

The results of this test show the maximum optical absorption intensity for the sample containing pure aptamer (0.2 µM) in the range of 260-265 nm. The highest concentration of cholecalciferol in its pure form, without the aptamer, exhibits the lowest peak intensity in the same range. In other samples, where the ligand-aptamer interaction has been established, the peak intensity of light absorption differs from that of the control samples, decreasing with increasing ligand concentration compared to the sample containing pure aptamer. However, all samples within the range between the two peaks described for the control samples exhibit an absorption peak (Fig. 2). These results demonstrate that the interaction between the aptamer and the target molecule induces the formation of a new secondary structure, leading to a shift in the absorbance intensity peak. The extent of these changes increases with the concentration of cholecalciferol as the target molecule.

Selection of a suitable buffer for the Fenton-like activity of MNPs

The type of buffer used influenced the peroxidase-like (Fenton-like) activity of MNPs. A comparison of the peroxidase-like activity of MNPs at a constant concentration across four different buffer types revealed that the acetate buffer at pH 4 exhibited the highest activity (Fig. 3).

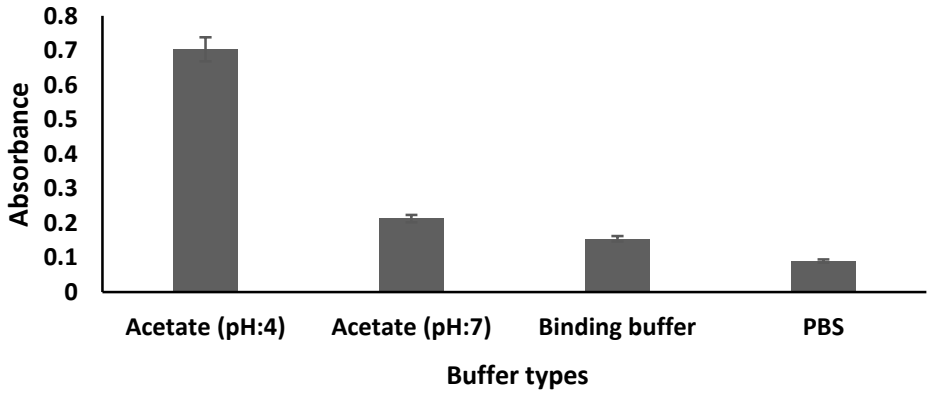

Fig 3. Optical absorption comparison chart: The obtained results show the effect of buffer type in a peroxidase-like reaction. The same concentration of MNPs was prepared in different buffers, then the peroxidase reaction was performed under the same conditions with the same ratios of chromogen and substrate.

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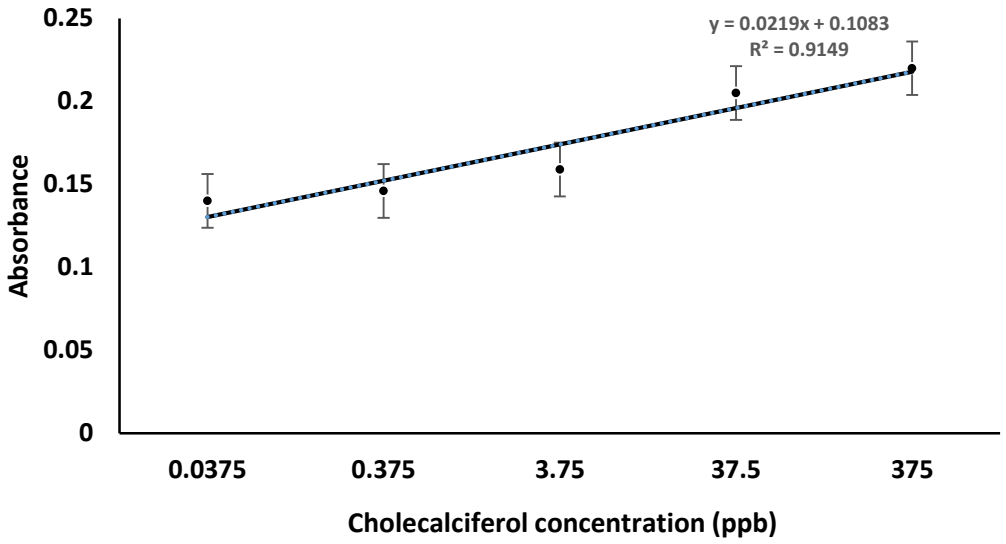

Fig 4. The standard curve describing the results obtained from the detection of serial dilutions of cholecalciferol with 10 nM aptamer

The results indicate that the peroxidase-like activity of the nanoparticles in the 0.2 M acetate buffer occurs more rapidly and at an earlier stage than in the other buffers. In contrast, PBS and binding buffer did not fully support the peroxidase-like activity of MNPs. Furthermore, while neutral acetate buffer provided suitable conditions for peroxidase-like activity, it was clear that the acidic acetate buffer was the most effective option.

Previous studies have explored the impact of various buffers on the surface charge of MNPs and their effects on the peroxidase-like activity of nanozymes. These studies consistently show that acidic acetate buffer is the most suitable for enhancing peroxidase-like activity and promoting a positive surface charge in bare MNPs [45, 46]. Other studies emphasize that peroxidase-like activity occurs only in an acidic buffer, while other enzymatic-mimicking behaviors, such as dehydrogenase-like activity, are exhibited in a neutral buffer. The findings also indicate that in a neutral acetate buffer, the magnetic nanozyme demonstrated significant peroxidase-like activity, despite unfavorable conditions. Overall, it can be concluded that an acidic buffer provides the most favorable conditions for the peroxidase-like activity of nanozymes; however, this activity is not limited to acidic conditions alone.

Development of detection assay

The detection of cholecalciferol was performed using a standard sample. The results showed that the absorption intensity during the detection of different concentrations of cholecalciferol with varying aptamer concentrations, while maintaining a constant concentration of MNPs, decreased as the concentration of Vitamin D3 increased, but only to a limited extent. None of the investigated aptamer concentrations provided satisfactory results. However, among these concentrations, the 10 nM aptamer (Fig. 4), which produced results with very narrow intervals, was selected as the optimal concentration for further studies with real samples.

Cholecalciferol detection in milk samples

Detection of cholecalciferol in milk was conducted in two modes. In the first mode, sample preparation was followed by previously established milk centrifugation techniques. Preliminary results indicate that the background color of milk significantly interferes with the signaling process, which relies on the peroxidase activity of MNPs (Fig. 5).

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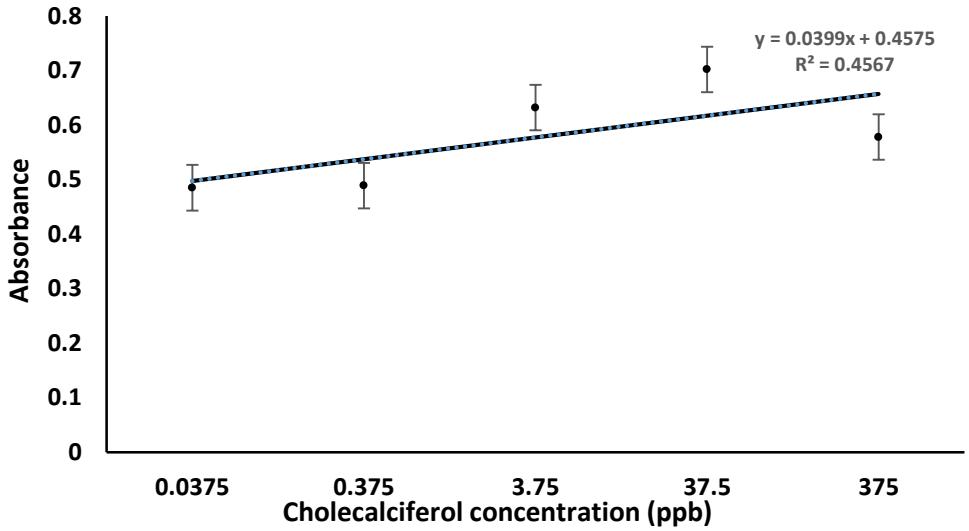

Fig 5. The standard curve of the obtained results for the detection of different concentrations of cholecalciferol in milk without removing the background color

On the other hand, while removing the background color using magnetic separation, the absorption values obtained in different samples did not significantly correlate with changes in Vitamin D3 concentration. The analysis of cholecalciferol in the concentration range of 0.0375 ppb to 375 ppb in this assay did not reveal a discernible trend in the results obtained from evaluating absorption intensity and color production (Fig. 6).

"The proposed nanozyme-based aptasensor for the colorimetric detection of Vitamin D3 in milk represents a significant advancement over previously established detection methods. A comparative analysis highlights differences across various parameters, including detection mechanisms, sensitivity, specificity, and practical applications.

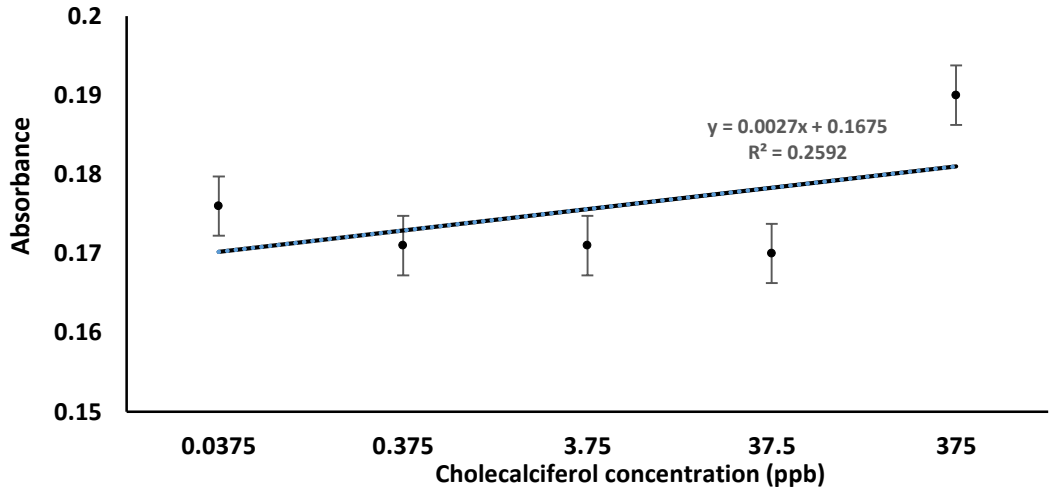

Fig 6. The standard curve of the obtained results for the detection of different concentrations of cholecalciferol in milk after removing the background color through magnetic separation

The nanozyme-based aptasensor utilizes magnetic nanoparticles (MNPs) as a colorimetric signal transducer. This system operates by altering the enzymatic activity of the nanozyme in response to the binding of Vitamin D3 to the aptamer, resulting in a quantifiable color change. In contrast, the ferrocene-tagged antibody method employs an electrochemical immunosensing platform that generates signals based on the electrochemical activity of ferrocene [37]. While effective for clinical samples, costly labeling techniques often limit this method. Impedimetric nano-immunochips rely on impedance measurements to quickly assess vitamin D deficiency [38], focusing on changes in electrical resistance rather than colorimetric variations, which may require more advanced instrumentation. The electrochemical immunosensor combining GCN-β-CD/Au nanocomposite materials enhances sensitivity through current changes instead of visual signals [39]. Additionally, the lanthanum nanoparticles-graphene quantum dots method employs complex nanomaterials for electrochemical sensing, concentrating on impedance and conductivity changes, which may not be as user-friendly as colorimetric approaches [40].

Regarding sensitivity and limit of detection (LoD), the nanozyme-based aptasensor demonstrates high sensitivity with a low LoD, making it effective for monitoring Vitamin D3 levels in milk samples. The ferrocene-tagged antibody method offers moderate sensitivity but may fall short in real-world applications due to its reliance on specific antibodies and intricate sample preparation. While impedimetric nano-immunochips deliver rapid results, their sensitivity may not match colorimetric methods [41]. The electrochemical immunosensor achieves commendable sensitivity [42]; however, its complexity could hinder routine testing applications. Similarly, although the lanthanum nanoparticles-graphene quantum dots method shows good sensitivity, its complicated fabrication may limit practical usage.

"Specificity is another critical factor in which the nanozyme-based aptasensor excels, due to the selective binding characteristics of aptamers, which reduce cross-reactivity with other substances found in milk. In contrast, while the ferrocene-tagged antibody method exhibits specificity, it may encounter cross-reactivity issues depending on the antibodies used, potentially leading to false positives [37]. The specificity of impedimetric nano-immunochips depends heavily on the immobilization process and the quality of antibodies used, which can vary between batches. The electrochemical immunosensor shares similar specificity concerns as other antibody-based methods but benefits from the stability provided by nanocomposites [38]. Finally, while generally high, the specificity of lanthanum nanoparticles-graphene quantum dots can be influenced by similar molecules in complex samples like milk [40].

Regarding application and practicality, the nanozyme-based aptasensor is designed for straightforward implementation in food safety and quality control, particularly in dairy products such as milk, making it well-suited for field testing scenarios. In contrast, the ferrocene-tagged antibody method is more appropriate for clinical environments due to its complexity and associated costs. Impedimetric nano-immunochips are ideal for rapid diagnostics but typically require specialized equipment that may not be available in all settings [38]. Although effective, the electrochemical immunosensor's complexity limits its practicality for routine testing outside laboratory environments [39]. Finally, while effective in theory, the complicated fabrication process associated with lanthanum nanoparticles-graphene quantum dots restricts their widespread application [40].

This comprehensive comparison highlights the advantages and limitations of various detection methods for vitamin D3 in milk, emphasizing how the nanozyme-based aptasensor is a promising alternative for effective monitoring in food safety contexts. However, our findings suggest that the magnetic separation process is considerably more practical and easier for removing the background color of the sample compared to other milk sample treatment methods (Table 1).

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Table 1. Statistical parameters of nanozyme-based aptasensor for detection of vitamin D3 in milk

Nanozyme-based aptasensor	R^2	LoD (ppb)	Minimum Absorbance
with optimized concentration of MNPs and aptamer	0.9194	0.0375	0.14 ± 0.01
without removing the background color	0.4567	0.0375	0.48 ± 0.05
with removing the background color	0.2592	0.0375	0.17 ± 0.005

Milk sample preparation in previous studies was complex and involved multiple steps, such as deproteinization and mechanical separation by centrifugation. In contrast, magnetic separation was performed here with high efficiency and simplicity, leveraging the magnetic properties of magnetic nanoparticles. This method reduced the complexity of the diagnostic process, shortened the overall procedure, and lowered costs. Additionally, using iron oxide nanoparticles, which serve dual purposes as magnetic nanozymes, offers another advantage. This approach simplifies the detection process by eliminating the need for peroxidase enzymes, reducing the cost of detecting the target molecule.

CONCLUSIONS

The nanozyme-based aptasensor for colorimetric detection of Vitamin D3 in milk stands out due to its simplicity, rapid results, high sensitivity, and specificity, making it particularly suitable for food applications. In contrast, traditional methods often require complex setups or are better suited for clinical diagnostics. The advancement towards colorimetric detection marks a significant step in making Vitamin D3 monitoring more accessible and user-friendly. The most notable aspect of our findings is the change in the peroxidase-like activity of MNPs after their surface is coated with the aptamer, compared to naked nanoparticles. By binding the aptamer to the MNPs, the surface coating reduces the rate of peroxidase-like reactions, resulting in lower chromogen oxidation rates. Our results demonstrated that the aptamer-coated nanoparticles increase the oxidation rate of TMB, directly affecting the peroxidase-like activity. Additionally, while the presence of cholecalciferol did not directly impact peroxidase-like activity, it may influence the activity indirectly by binding to the aptamer, potentially altering the color signal. Ultimately, the optimized platform did not yield technically descriptive results for detecting cholecalciferol in milk. It can be assumed that other factors in the detection process must be optimized, a consideration not addressed in the present study.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

Ethical approval for this study was obtained from the Research Ethics Committees of Mazandaran University of Medical Sciences (IR.MAZUMS.REC.1401.451).

AUTHORS CONTRIBUTION

Abolghasem Rahmani (A.G.R.) data curation; formal analysis; investigation; methodology; software; validation; writing – original draft. Adele Rafati (A.R.) conceptualization; investigation; supervision; writing, review, and editing. Zahra Valipanah (Z.V.) conceptualization; validation; writing, review, and editing. Pooria Gill (P.G.) conceptualization; funding acquisition; project administration; resources; supervision; writing, review, and editing.

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