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Isolation of Nucleic Acids from Different Biological Objects with Silica–Magnetite Nanoparticles

N. N. Volkova, O. N. Derjabin, and N. O. Dudchenko

*Institute for Applied Problems of Physics and Biophysics, N.A.S. of Ukraine,
3 Sluzhbova Str.,
03142 Kyiv, Ukraine*

Now, modified magnetic particles are widely used in different biological and medical applications (enzyme and protein immobilization, cells separation and purification, MRI, targeted drug delivery, etc.). The aim of the present study is to reveal the ability of synthesized silica-modified magnetic particles to isolate DNA from the biological tissues in comparison with common method used the non-magnetic particles. Magnetite (Fe_3O_4) particles are prepared via co-precipitation of Fe^{+2} and Fe^{+3} with NH_4OH in aqueous solution. Silica–magnetite nanocomposites are prepared via tetraethoxysilane hydrolyzation in alcohol–water–ammonia mixture. The average core size of synthesized magnetic nanoparticles is about 15 nm (according to the TEM data). Application of these compounds for DNA isolation from different biological objects showed significant time-savings, overall higher yields, lower RNA contamination and better polymerase chain reaction (PCR) amplification compared to commercial available silica non-magnetic particles (Promega). High efficiency of nucleic-acid purification by silica–magnetite particles is confirmed in molecular assays with reverse transcriptase–polymerase chain reaction (RT–PCR) assays of RNA- and DNA-virus diseases of plants, avian, cattle and estimation of bacterial spectrum in dairy products (probiotics).

Модифіковані магнетні частинки зараз широко використовуються для різних біологічних та медичних застосувань (імобілізація ензимів та білків, виділення та очищення клітин, ЯМР, направлена доставка ліків та ін.). Метою цього дослідження було показати здатність синтезованих магнетних частинок, модифікованих кремнеземом, виділяти ДНК з різних біологічних тканин у порівнянні із стандартною методою з використанням немагнетних частинок. Магнетитові (Fe_3O_4) частинки було одержано шляхом співосадження Fe^{+2} та Fe^{+3} за допомогою NH_4OH у водній розчині. Силіка-магнетитові наноконізити були одержані шляхом гідролізації тетраетоксисилана у спирто-водяно-амонійній суміші. Середній розмір ядра синтезованих магнетних наночастинок був біля 15 нм (за даними просвітлювальної електронної мікроскопії). Застосування цих сполук для виділення ДНК з

різних біологічних тканин виявило значне заощадження часу, загальний більш високий вихід ДНК, більш низьку кількість домішок РНК та кращу ампліфікацію полімеразної ланцюгової реакції (ПЛР) у порівнянні з традиційними силіційовими немагнетними частинками (Promega). Високу ефективність очищення нуклеїнових кислот за допомогою силіка-магнетитових частинок було підтверджено в молекулярно-біологічних тестах, що були виконані на основі зворотної транскрипції з подальшою полімеразною ланцюговою реакцією (ЗТ-ПЛР) щодо виявлення РНК та ДНК вірусних захворювань рослин, птахів, сільськогосподарських тварин та оцінки якості молочних продуктів (пробіотиків).

Модифицированные магнитные частицы сейчас широко используются в различных биологических и медицинских приложениях (иммобилизация энзимов и белков, выделение и очистка клеток, ЯМР, направленная доставка лекарств и т.д.). Целью настоящего исследования было показать способность синтезированных магнитных частиц, модифицированных кремнеземом, выделять ДНК из различных биологических тканей по сравнению со стандартным методом с использованием немагнитных частиц. Магнетитовые (Fe_3O_4) частицы были получены путем соосаждения Fe^{+2} и Fe^{+3} с помощью NH_4OH в водном растворе. Кремний-магнетитовые наноконпозиты были приготовлены путем гидролиза тетраоксисилана в спирто-водно-аммониевой смеси. Средний размер ядра синтезированных магнитных наночастиц был около 15 нм (по данным просвечивающей электронной микроскопии). Применение этих соединений для выделения ДНК из различных биологических тканей выявило значительную экономию времени, общий более высокий выход ДНК, более низкое количество примесей РНК и лучшую амплификацию полимеразной цепной реакции (ПЦР) по сравнению с коммерчески доступными кремневыми немагнитными частицами (Promega). Высокая эффективность очистки нуклеиновых кислот с помощью кремний-магнетитовых частиц была подтверждена в молекулярно-биологических тестах, выполняемых на основе обратной транскрипции с последующей полимеразной цепной реакцией (ОТ-ПЦР) по выявлению РНК и ДНК вирусных заболеваний растений, птиц, сельскохозяйственных животных и оценке качества молочных продуктов (пробиотиков).

Key words: silica-magnetite nanoparticles, DNA isolation, magnetic particles.

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1. INTRODUCTION

The superior sensitivity of nucleic acid amplification technique enables diagnosis of infectious diseases an early stage before positive serologic results confirm an infection. These molecular methods have become a standard application in clinical laboratory in recent years. In addition to diagnosis of infectious diseases, the determination of virus load has gained increasing importance in medical and veterinarian virology laboratory. Although the introduction of real-time polymerase

chain reaction (PCR) has led to considerable progress in automating the amplification and detection steps of molecular biological technique, a nucleic acid isolation remains very labour-intensive when performed with traditional phenol–chloroform extraction and ethanol precipitation methods. Additionally, these methods are too complicated, time-consuming, and hazardous and produce on the last stage denaturated nucleic acids.

Modified magnetic particles now are widely used in different biological and medical applications (enzyme and protein immobilization, cells separation and purification, MRI, targeted drug delivery, *etc.*) [1, 2]. Due to the strong magnetic properties and low toxicity of magnetic particles, their applications in biotechnology and medicine have gained significant attention. Basically, all types of magnetic particles consist of magnetic core with inorganic or organic shell. The target molecules or cells are captured on magnetic particles coated with a target-specific surface, and separated from unbound components by the application of magnetic field. The need for quick bacterial plasmid DNA and virus DNA/RNA preparation methods has increased the flood molecular protocols requiring highly purified genetic templates [1–3].

Magnetic separation of DNA offer benefits over usual method due to rapid processing time, reduced chemical needs, the ease of separation [1]. Thus, the aim of the present study was to reveal the ability of synthesized silica-modified magnetic particles to isolate DNA from different biological tissues in comparison with common method based on a non-magnetic sorbents.

2. MATERIALS AND METHODS

Materials. Ferric chloride hexahydrate, ferrous sulphate tetrahydrate, tetraethoxysilane were purchased from Sigma Chemical Co. Agarose L (low electroendosmoid) was from Amersham Biosciences (Uppsala, Sweden). Reagents for use in DNA isolation and analysis were of molecular biology grade. Ribonuclease A was obtained from 'Sigma'. All other chemicals and solvents used were of analytical grade. The water used throughout this work was the reagent-grade water produced by Milli-Q Ultra-Pure-Water Purification System.

Preparation of Silica–Magnetite Nanocomposites. The magnetite particles were prepared via co-precipitation of Fe^{+2} and Fe^{+3} with NH_4OH in aqueous solution under normal conditions. Stock solutions of 1 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and 2 M $\text{FeSO}_4 \cdot 4\text{H}_2\text{O}$ were prepared as a source of iron by dissolving the respective chemicals in deionised water under stirring. Stock solution of 1 M NH_4OH was prepared by dilution of concentrated NH_4OH solution. The reagents solutions were mixed quickly in reaction vessel, and 50 ml of ammonium solution was added drop-by-drop to reaction mixture under slow mechanical stirring. After the re-

action completing, magnetic particles were lightly dispersed using ultrasound disperser, three times rinsed with deionised water to remove the residual surfactant and unreacted reagents.

Obtained magnetite was coated with silica via tetraethoxysilane hydrolyzation in alcohol–water–ammonia mixture. Thereto, obtained magnetic particles were dispersed in 25 ml of water using ultrasound disperser. 100 ml of ethanol, 2 ml of concentrated NH_4OH were added to the reaction mixture at slow mechanical stirring. After that, 3 ml of tetraethoxysilane (TEOS) were added drop-by-drop to the reaction mixture. The hydrolysis of TEOS was carried out for 20 hours under normal conditions.

The resultant product was thoroughly rinsed with deionised water three times to remove the residual surfactant and unreacted reagents, and collected by magnetic separation using a permanent magnet. The silica–magnetite nanocomposite (MAGNAT) was stored in deionised water at a concentration of 10 mg/ml.

Characterization of Magnetic Nanoparticles. The size and morphology of magnetic nanoparticles were observed by transmission electron microscopy (TEM) using PEM-U (Sumy, Ukraine). Magnetic measurements were performed using magnetometer with Coulomb sensor (Tver University, Russia). X-ray diffraction measurements performed using diffractometer DRON-UM1 in filtered emission CoK_α with recording Bragg–Brentano geometry.

Magnetic Response Characteristics. Magnetic response of synthesized magnetite nanocomposites was measured by monitoring an optical density of the magnetite adsorbent suspended in water at 600 nm. A spectrophotometer cuvette holder with attached neodymium (S36 grade) magnet was used.

Binding Capacity of Magnetite Nanocomposites. Binding capacity of engineered nanoparticles was tested against Marker DNA standards with different molecular mass. Binding and recovery of Marker DNA fragments (λ DNA/*Hind* III with 125–23.130 bp and ϕ X174/*Hae* III with 72–1.353 bp) were titrated into 2000 μg concentrations of nanoparticles and nonmagnetic commercial absorbent. Binding was performed in binding buffer for DNA purification, elution of absorbed nucleic was carried out in deionised water. Eluted DNA was quantified by absorbance at 260 nm.

Purification of Plasmid DNA by Silica–Magnetite Nanocomposites. *E.coli* cells expressing the plasmid pGL3-Conrol-Vector were grown to log phase in culture media containing 100 $\mu\text{g}/\text{ml}$ ampicillin. Bacterial cells were harvested from 3 ml of cell culture and treated with 0.05 M Tris-HCL $\mu\text{g}/\text{ml}$ ribonuclease A. Cell lysis was performed with 0.2 M NaOH containing 1% of dodecil sulphate. Genomic DNA and other contaminants were precipitated by addition of 6M guanidine-hydrochloride, pH 5.5. After centrifugation, the cell lysates were used for plasmid DNA purification with synthesized nanocomposites. Binding and elu-

tion of plasmid DNA were performed with common procedure and chemicals. The concentration of purified nucleic acids was calculated using absorbance at 260 nm.

Purification of Total DNA from Plants, Bacterial and Mammal Tissues by Silica–Magnetite Nanocomposites. Procedures of DNA extraction from different biological samples were the same as it was written above but included the stage of tissue homogenization before DNA extraction and usage of tissue specific buffer systems.

Isolation of Total DNA from Milk Food Products by Silica–Magnetite Nanocomposites. Procedure of DNA extraction from milk product samples were the same as it was written as it is indicated above.

Total RNA Extraction by Silica–Magnetite Nanocomposites from Avian and Mammal Tissue Samples. Purification of total RNA from avian tissue, embryonated eggs and porcine blood cells were performed with usage a commercial buffer system kits for RNA extraction ('AmpliSens', Russia). Mononuclear blood cells were harvested by centrifugation and washed two times with Hank's solution. Cell pellets were mixed with extracting buffer and suspension of silica–magnetite nanoparticles in dose of 10 µl (stock concentration 10 mg/ml) were added to each samples. Sedimentation was carried out by neodymium (S36 grade) magnet ('Promega', USA). Repeated procedure of suspension/sedimentation was made. Elution of absorbed nucleic acids was carried out in deionised water. Eluted RNA was quantified by absorbance at 260 nm. All above manipulations were done in 4°C. Purified RNA immediately was used for reaction of reverse transcription.

Reverse Transcriptase Reaction (a Single cDNA Synthesis). Reaction mixture included 1 µg of total RNA, 0.5 µg oligo dT₁₈ primer and incubated in microtubes for 5 min in 70°C, cooled on ice. Then 1 mM dNTP, 10 mM Tris buffer, 40 units of RNase inhibitor were added to each reaction mixture and 5 min incubation in 37°C was followed. Finally, 200 units of M-MuLV enzyme were added to reaction cocktail and it was incubated for 60 min in 37°C. Then, inactivation of enzyme was made during 10 min in 70°C. Synthesized cDNA was stored in –20°C.

PCR Amplification. The PCR procedure had been carried out with primers targeting the insertion elements IS900 of Map. The mass of amplicons is 800 bp. The purified DNA (0 µl) was mixed with cocktail including PCR buffer (10 mM Tris-HCL, pH 8.3) with 50 mM KCL, 1.5 mM MgCL, 5.0 pm/ml of each primers, 200 µM of each dNTP, 2.5 U Tag DNA polymerase. Cycling conditions were 20 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 1 min. Molecular mass of amplified DNA fragments were detected by electrophoresis in 1% agarose with 0.5 µg/ml ethidium bromide and 1 kb molecular mass standards (Sigma). The running buffer was TAE (49 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). Electrophoresis was carried out at 90 V for 1 hour. Visualisation PCR products was performed by UV illumination.

3. RESULTS AND DISCUSSION

The size and morphology of magnetic particles were characterized by TEM (data not shown). It shows that the size of magnetic nanoparticles is about 15 nm. X-ray diffraction (XRD) measurements show that the magnetic core of the synthesized particles consists of magnetite (Fe_3O_4). Six characteristic peaks for Fe_3O_4 in XRD pattern (data are not shown) were observed for magnetic nanoparticles. These peaks reveal that the resultant particles were pure Fe_3O_4 .

The superparamagnetic properties of the magnetic particles were verified by magnetization curve measurements. Saturation magnetization of silica-modified magnetite particles was 37 emu/g ($\text{A}\cdot\text{m}^2/\text{kg}$). This saturation magnetization of magnetic particles makes them susceptible to magnetic field and therefore makes the solid and liquid phases separate easily.

Magnetic response of silica–magnetite nanoparticles has been analysed. Magnetic response was measured by placing nanoparticles in buffer solution in spectrophotometer cuvette attaching to magnet on its outside wall. Optical density of particles suspensions was measured at 600 nm over time. More than 90% of magnetic nanoparticles at concentration above 0.1 mg/ml were removed from buffer solution in less than 10 seconds after magnetic field applying. Sedimentation of 90% of nanoparticles at concentration 0.01 mg/ml were observed 25 seconds after applying of magnetic field.

The mechanism of this process could be envisaged in the following way. At the first stage of sedimentation, a few particles magnetize and self-attract to form a critical particle mass that moves toward magnet. At the required particle concentration for most molecular-biological applications, efficient removal of particles is accomplished in under 30 seconds (Promega DNA kits). In case of our nanoparticles, about 15 seconds is enough for optical clearing of solution. The standard variant of magnet was used for these investigations (Promega's MagneSil™ magnetic stand, which incorporates S36 grade neodymium rare earth magnet).

Binding Capacity of Magnetite Nanocomposites. Ionic strength and pH are the crucial factors estimating processes binding and elution of nucleic acids by silica magnetite nanobeads. Absorption capacity of nanoparticles could be modulated in wide ranges by the ionic strength of binding buffer system, which is used for DNA purification. DNA is a polyanionic molecular due to presence of phosphate groups and interacts with positively charged functional groups on silica–magnetite particles surface [3]. In order to determine the ion strength effect on synthesized nanocomposites, we tested several NaCl concentration ranges of 0–4 M in binding buffer. It had been found that presence of 2M NaCl and above concentrations resulted in maximal binding of

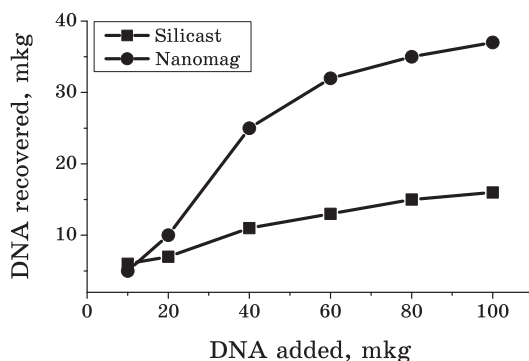


Fig. 1. Recovery of small (72-1.353 bp) marker DNA fragments from magnetic nanocomposites and commercial silica adsorbent.

plasmid DNA and marker small DNA fragments. In this study, the influence of binding buffer pH on DNA absorption of nanocomposites had been estimated also. As expected, pH of binding solution had no effect on plasmid DNA absorption by silica-magnetite nanoparticles. These results are in agreement with data obtained by Chen-Li Chiang [1, 6] for silica-magnetite nanoparticles with more diameter size (about 31 nm).

As revealed, the synthesized nanoparticles possessed an increased recovery small DNA marker fragments over commercial nonmagnetic materials (Fig. 1). This recovery was inversely related to DNA size and much higher recovery of smaller marker DNA was registered. Final recovery of small fragments was a primary function of binding since these DNA were efficiently eluted from nanocomposites in water. The recovery of lambda marker DNA fragments was a function of both binding capacity and elution efficiency of synthesized nanocomposites. The larger fragments of DNA have a reduced binding capacity at these conditions and reduced efficiency of elution in water at room temperature.

Plasmid DNA Purification by Silica-Magnetite Nanocomposites. Obtained magnetic particles were tested for DNA isolation from *E.coli* cultures, which had been transfected with some gene-engineering constructions. A set of experiments with bacterial cell lysates for measuring of absorption capacity of synthesized nanoparticles were performed. The increasing amounts of nanoabsorbent were added to bacterial cell lysates prepared from 10 ml cultures of *E.coli* containing the high copy number of pGL3-Control Vector (plasmid DNA). The traditional silica adsorbent of nucleic acids was used as a control. The result of silica magnetite nanoparticles usage was an isolation of 80 μ g of plasmid DNA at 2.2 mg particles added to lysate. In contrast, 5.0 mg commercial tradition silica adsorbent was required for isolation of equivalent amount of plasmid DNA from a 10 ml culture. The results demonstrated that absorbing capacity of magnetic particles was sig-

nificantly higher than that of traditional non-magnetic silica carriers. ***Application of Silica-Magnetite Nanoparticles in Molecular Diagnosis of Cattle and Avian Viral Diseases.*** The worldwide occurrence and re-occurrence of trans-boundary viral diseases like classical swine fever indicates that there is an acute need for the development of high-capacity, powerful and reliable methods for detecting a causative viral and bacterial agent before they could spread to large populations and cause a tremendous loss. During the last one and a half decade, more than 40 nested polymerase chain reaction assays have been developed for variety of DNA and RNA viruses. False negative and positive results are avoided now by the using of special tools, practices and internal controls for purification nucleic acids and technique of amplifications.

In this study, we tested the possibility of synthesized nanocomposites for high-effective purification of native total RNA from porcine and avian tissues for diagnosis of avian Bronchitis virus and virus of classic swine fever in domestic and wild populations [1, 2]. Results of reverse transcriptase-polymerase chain reaction (RT-PCR) for molecular diagnosis of viral diseases are presented in Fig. 2. The same results were found in RT-PCR assay of health and infected avian an embryonated eggs by bronchitis virus [1, 2].

Comparative analysis of data obtained with usage of silica-magnetite nanocomposites and commercial silica absorbent demonstrated a reduction of false negative samples in diagnosis these viral diseases and made procedure of RNA purification much more easy, fast and simple.

Application of Silica-Magnetite Nanoparticles in Molecular Diagnosis of Anthrax. *Bacillus anthracis* is the etiologic agent of anthrax, an acute fatale disease among mammals. It was thought to differ from

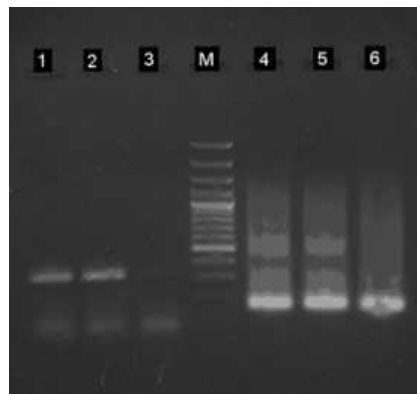


Fig. 2. The agarose gel electrophoresis of the RT-PCR products, which were obtained from tissues of health and infected swine. Lines 1, 2, 3—health animals; M—DNA markers; lines 4, 5, 6—amplified fragments of viral DNA.

Bacillus cereus, an opportunistic pathogen and cause of food poisoning, by presence of plasmids pXO1 and XO2, which encode the lethal toxin complex and poly-*D*-glutamic acid capsule, respectively.

In this set of experiments, the silica-magnetite nanoparticles were used in differential molecular diagnostic of *Bacillus anthracis* and non-*B-anthraxis* bacteria. With PCR-based technique it was confirmed the presence of both plasmids which encode capsule and toxin of *Bacillus anthracis* in one bacterial strain which earlier was conceded as *Bacillus cereus* and was known as weak pathogen (Fig. 3).

This work confirmed the fact that non-*B-anthraxis* bacteria could possess the anthrax toxin genes and explained their high pathogenic



Fig. 3. The agarose gel electrophoresis of the PCR amplified DNA fragments from bacterial vaccine strains, *Bacillus cereus (anthracoides)* and bacterial vaccine strains with protective antigen. Lines from 1 to 8—vaccine strains (weak immunogens); line 9—*Bacillus anthracoides* within capsule; line M—DNA markers; lines from 10 to 13—vaccine strains with protective antigen (high immunogenic properties).

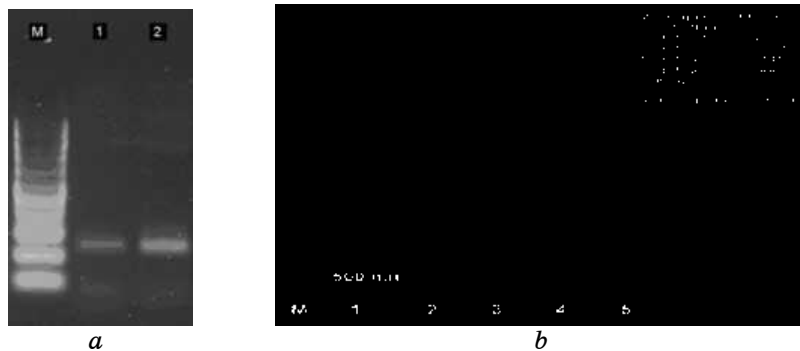


Fig. 4. The agarose gel electrophoresis of the PCR amplified virus DNA fragments from sugar beet (a) and *Bifidobacterium* DNA from probiotic tablets (b). Line M—DNA markers; line 1—commercial absorbent; line 2—magnetite nanoparticles. Line M—DNA markers; line 1—negative control; lines from 2 to 5—*Bifidobacterium* strains.

properties in causing a severe inhalation anthrax-like illness. Therefore, the presence of amplified DNA fragments with molecular mass of capsule antigen has proved the virulence of this bacteria strain and a potential dangerous of some vaccine drugs made on base of *Bacillus anthracoides* for personals and cattle.

Isolation and Identification of DNA in Plant and Dairy Products. In this experimental set, the nanoparticles were hydrolyzed and carboxyl groups on there surface were inducted by oxidation. The carboxyl-functionalized silica-magnetite nanocomposites were tested for binding of *Bifidobacterium* and *Lactobacterium* DNA from crude lysates of different probiotic tablets or from culture cell lyophilisates [1]. The binding capacity of nanoparticles was higher then traditional commercial silica absorbent. The efficiency of DNA purification was confirmed by results of PCR amplification with specific primers for these bacterial strains (Fig. 4, b).

Comparative analysis of viral DNA detection in sugar beet was performed with usage of commercial nonmagnetic and synthesized silica-magnetite nanoparticles. As it followed from data performed in Fig. 4, a, the binding capacity and efficiency of tested nanocomposites were much more high then traditional DNA absorbent.

Thus, the data obtained have proved the high efficiency of synthesized silica-modified magnetite particles for high purification of vital and bacterial native DNA/RNA for nucleic acid amplification technique.

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