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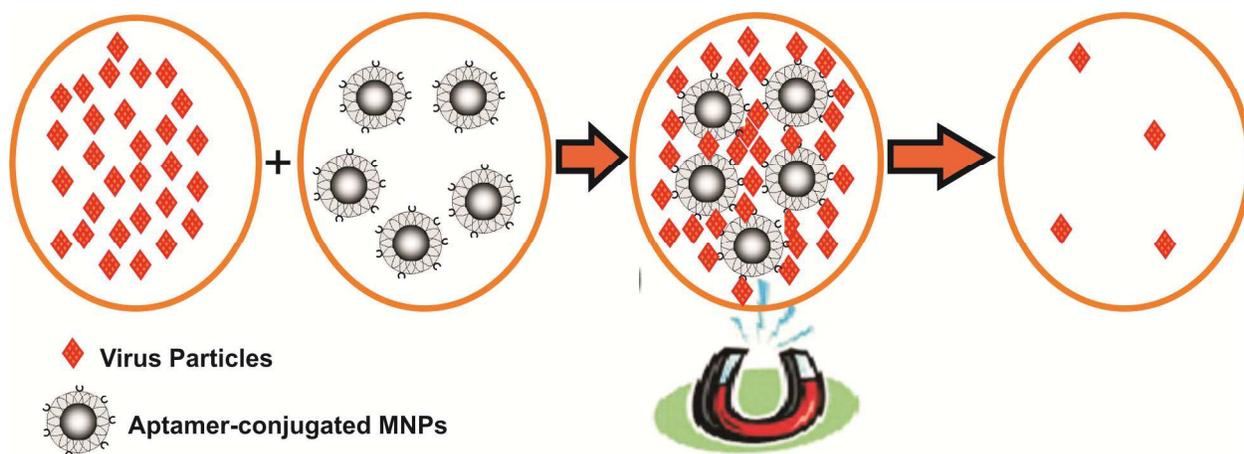


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Described here a methodology for selective capturing HCV particles from human plasma samples using aptamer-conjugated magnetic nanoparticles. The aptamers were specific for binding to E1E2 glycoprotein of HCV viruses.

## **Aptamer-conjugated Magnetic Nanoparticles for Efficiently Removal of HCV Particles from Human Plasma Samples**

Najmeh Delaviz <sup>1</sup>, Pooria Gill <sup>2\*</sup>, Abolghasem Ajami <sup>1</sup>, Mohsen Aarabi <sup>3</sup>

1. Molecular Cell Biology Research Center, Mazandaran University of Medical Sciences, Sari, Iran

2. Nanomedicine Group, Immunogenetics Research Center, Mazandaran University of Medical Sciences, Sari, Iran

3. Diabetes Research Center, Mazandaran University of Medical Sciences, Sari, Iran

### **\*Corresponding Author**

Pooria Gill; M.Sc., Ph.D. of NanoBiotechnology

Nanomedicine Group, Immunogenetics Research Center, Mazandaran University of Medical Sciences, Sari, Iran

Email Addresses: [pooriagill@yahoo.com](mailto:pooriagill@yahoo.com); [p.gill@mazums.ac.ir](mailto:p.gill@mazums.ac.ir)

Tel/Fax: +98-11-33261247; Mobile Tell: +98-9127615950

### **Running Title**

Aptamer-conjugated MNPs for HCV removal of plasma

**Abstract**

Described here a methodology for selective capturing HCV particles from human plasma samples using aptamer-conjugated magnetic nanoparticles. The aptamers were specific for binding to E1E2 glycoprotein of HCV viruses. The nanoconjugate was confirmed using UV-visible and FTIR spectroscopies; however, the transmission electron micrographs demonstrated efficiently capturing of these nanoconjugates within a magnetic force. Also, the quantitative measuring of HCV particles in human plasma samples by Real-time PCR confirmed efficiently removal of the viral load.

## 1. Introduction

The hepatitis C virus is a single stranded RNA virus that belongs to the family of flaviviruses, affecting about 3% of the human population (1-3). Chronic HCV is the primary cause of cirrhosis, hepatocellular carcinoma (HCC), and end-stage liver disease (4,5).

Until recently, the standard of care for patients with chronic hepatitis C involved dual therapy with pegylated interferon (IFN) alpha and Ribavirin (PEG IFN/riba) and recently approved protease inhibitors(6,7). Conventionally, the eradication of HCV and a sustained status of undetectable HCV-RNA have been regarded as the most important factors for obtaining better clinical results after IFN therapy. Low viral load may predict lower recurrence and better survival in patients undergoing hepatic resection for HCV-related HCC (8).

It has been previously shown that aptamers are single-stranded oligonucleotides, DNA or RNA, with the ability for binding to a broad range of targets, such as peptides, proteins, drugs, organic and inorganic molecules, or even whole cells and viruses based on their specific binding pockets for the target molecule, with high affinity and specificity (9-11). The combination of aptamers with novel nanomaterials, i.e. nanomaterial-based aptamer bioconjugates, has attracted considerable interest and led to a wide variety of applications in medicine.

Superparamagnetic iron oxide nanoparticles, including magnetite ( $\text{Fe}_3\text{O}_4$ ) and maghemite ( $\text{g-Fe}_2\text{O}_3$ ), have been a major research focus during the past decade

(12,13). Aptamer-functionalized MNPs (Apt–MNPs) have been used for detection of small molecules, pathogens, viruses and cells (14,15). In addition, It had been previously reported DNA aptamer sequences for targeting envelope protein of HCV viruses (16,17). Here, the magnetic nanoparticles have a magnetic core, coated with starch, and were conjugated to a synthetic DNA aptamer against HCV E1E2 envelope glycoprotein (Figure 1). By using this nanoconjugate, we demonstrated here a methodology for *in vitro* capturing of HCV viruses from human plasma and efficiently lowering viral load (Figure 2) assessed by quantitative real-time PCR.

<<Figure 1>>

<<Figure 2>>

## 2. Results and discussion

### 2.1. UV-Visible and FTIR spectra

Figure 3 indicated the UV-Vis spectra of MNPs after and before conjugation to amino-modified aptamers. MNPs demonstrated a peak at  $380 \pm 5$  nm using UV-Vis spectroscopy. Also, aptamers showed a peak at 260 nm; however, two peak at 260 nm and  $380 \pm 5$  nm were seen from aptamer-conjugated MNPs. These two peaks confirmed efficiently interaction between amino-modified aptamers and starch functionalized MNPs.

<<Figure 3>>

Figure 4 revealed FTIR spectra of starch-functionalized MNPs before and after covalent binding to amino-modified aptamers. Starch-functionalized MNPs showed four peaks at 3150, 1650, 1400, and 1050  $\text{cm}^{-1}$ . The absorption regions observed at 3150 and 1650  $\text{cm}^{-1}$  were due to the presence of water molecules, and the peak at 1400  $\text{cm}^{-1}$  reflects the bending modes of O-C-H, C-C-H, and C-O-H angles. The absorption region at 1050  $\text{cm}^{-1}$  relates to C-C and C-O stretching modes of the polysaccharide backbone. The absorption at 1400  $\text{cm}^{-1}$  confirmed covalent binding of amino-modified aptamers to starch-functionalized MNPs through a hydroxyl group.

<<Figure 4>>

## 2.2. Real time PCR

Remaining HCV particles were measured using quantitative real-time RT-PCR (Q RT-PCR) as standard method for HCV RNA detection (18). The viral load of four positive plasma were determined as  $11175000 \pm 2250$  (Sample 1),  $2547000 \pm 750$  (Sample 2),  $406666 \pm 250$  (Sample 3), and  $2623666 \pm 10500$  (Sample 4) IU/ml. After interaction with the aptamer-conjugated MNPs, the load of HCV particles were significantly reduced. Table 1 showed the comparative HCV viral loads before and after being challenged with the aptamer-conjugated magnetic nanoparticles; however, no viral load changes were seen from MNPs with no aptamer, after challenging by positive samples.

<<Table 1>>

## 2.3. TEM

Particle size and morphology of aptamer-conjugated magnetic nanoparticles were revealed using transmission electron microscope (TEM). It was demonstrated that MNPs (Figure 5-a) and the aptamer-conjugated MNPs (Figure 5-b) were well dispersed and spherical, with an average size 100 nm. Capturing of HCV viruses by the aptamer-conjugated MNPs was also confirmed by TEM. The TEM micrograph revealed many accumulated HCV particles ( $\sim 50$  nm each) captured by the aptamer-conjugated MNPs (Figure 5-c).

<<Figure 5>>

#### 2.4. DLS

Particle size of magnetic nanoparticles was measured via dynamic light scattering (DLS). It was revealed that MNPs (Figure 6, series 1) were well dispersed with a hydrodynamic size of ~100 nm. Capturing of HCV viruses by the aptamer-conjugated MNPs was also demonstrated by the particle size changes. Figure 6 (series 2) depicted two hydrodynamic sizes of nanoparticles after capturing HCV particles (~50 nm) by the aptamer-conjugated MNPs (~100 nm).

<<Figure 6>>

#### 2.5. Capturing efficiency of aptamer-conjugated MNP for HCV removal

By comparing the initial viral load of each sample before and after challenging by the magnetic nanoconjugates, the capturing efficiency of nanoconjugate were calculated. For this purpose, the initial viral load was divided to the load acquired after interacting with a defined concentration of aptamer-conjugated MNPs ( $5 \times 10^{-1}$ ,  $5 \times 10^{-2}$ ,  $5 \times 10^{-3}$ ,  $5 \times 10^{-4}$ , and  $5 \times 10^{-5}$   $\mu\text{g/ml}$ ). Decreasing of the capturing efficiency percentages by lowering concentrations of nanoconjugates in comparison to MNPs, demonstrated specifically removing of virus particles via the aptamer- conjugated MNPs (Table 1). Obtained results

indicated lowering viral loads after challenging with the higher concentrations of aptamer-conjugated MNPs. In addition, the highest concentrations of aptamer-conjugated MNPs could remove the viral particles with more than 91% capturing efficiency; that it could mean more than 91% detection dynamic range for this capturing methodology.

### 3. Experimental

#### 3.1. Chemicals and Instruments

Starch-functionalized iron oxide magnetic nanoparticles (fluidMAG) and magnetic separator plate were purchased from Chemicell (berlin, Germany). Amino group modified aptamers were synthesized by Bioneer Corporation (Korea). Hepatitis C virus real-time PCR kit for *in vitro diagnosis* and pathogen free RNA isolation kit were purchased from Gene Proof (Czech Republic). Cyanogen bromide, NaHCO<sub>3</sub>, phosphate buffer saline (PBS), bovine serum albumin (BSA) and sodium azide were obtained from Sigma-Aldrich, Germany. Real-time PCR assays were done using RotorGeneQ (Corbett Robotics, Qiagen). Transmission electron microscopy was done using Philips EM028 transmission electron microscope. FTIR spectra were taken using Fourier transform infrared spectroscope, Perkin Elmer, USA. UV-Vis spectra were given using UV-Vis spectrophotometer (WPA Biowave II, UK). The particle size distribution was measured using a Nano-ZS (red badge) ZEN 3600 (Malvern, UK).

#### 3.2. Specimens

Positive HCV Human plasma samples were collected from Sari Blood Transfusion Organization (Mazandaran Province, Iran). All samples were negative for hepatitis B surface antigen, and HIV antigens and antibodies. Western blot positive samples tested by real-time RT-PCR in this work.

### 3.3. Selection of aptamer

It has been previously reported many aptamers for HCV viruses (19). We selected the aptamer that it was against the envelope protein of hepatitis C virus (E1E2 glycoprotein), so-called as Apt-E1E2-6, showed a high affinity and blocked virus binding sites to the related cells. The Apt-E1E2-6 exerted its antiviral effects through inhibition of virus binding to host cells by blocking E1E2 glycoprotein (16).

### 3.4. Bioconjugation of aptamer to MNPs

For preparing aptamer-conjugated magnetic nanoparticles, the amino group-modified aptamers were coupled to hydroxyl group of the starch functionalized MNPs according to the manufacturer instruction. In brief, the fluidMAG nanoparticles were washed one time with 1 ml activation buffer (0.2 M Sodium hydrogen carbonate buffer, pH 8.4), then using the magnetic separator and resuspend the particles in 0.25 ml activation buffer. 0.05 ml CNBr was added to the nanoparticles and mixed by vortexing. The tube was placed in ice cold water for 10 minutes. The resulting nanoparticles were washed quickly 2 times with 1 ml PBS and then the activated nanoparticles were dispersed in 0.25 ml PBS. The amino group containing aptamers were added to the activated nanoparticles and mixed the suspension on a shaker for 2 hours at room temperature and then the nanoparticles were washed 3 times with 1 ml PBS, and finally the

nanoparticles were re-suspend in blocking/storage buffer (PBS, 0.1 % BSA, 0.05 % sodium azide) by vortexing.

### **3.5. UV-Visible spectroscopy of aptamer-conjugated MNPs**

The UV-Vis absorption spectra of the MNPs and aptamer- conjugated MNPs were obtained using Helma tray cell covet. For each sample, a droplet (volume of 3 $\mu$ l) was used and the spectrum saved using Biochrome PVC software.

### **3.6. FTIR spectroscopy of aptamer-conjugated MNPs**

Aliquots of MNPs and the prepared nanoconjugates were prepared in PBS. From each sample, 5 ml were dried in room temperature for 2 hours. The dried powders were collected, grind with potassium bromide (KBr) and compressed as disks. After compression, each disk was put into a Fourier transform infrared spectroscope, with a digital band analysis and analyzed. The chemical bonding information on metaloxygen, hydroxyl, and other functional groups were obtained by use of Fourier transform infrared spectroscopy. Each spectrum was collected after 32 scans at a resolution of 1  $\text{cm}^{-1}$ .

### **3.7. Removal of HCV particles using aptamer-conjugated MNPs**

Ninety microliter of the prepared nanoconjugates (with the concentrations  $5 \times 10^{-1}$ ,  $5 \times 10^{-2}$ ,  $5 \times 10^{-3}$ ,  $5 \times 10^{-4}$ , and  $5 \times 10^{-5}$  mg/ml) were added to the equally volume of plasma samples with the determined HCV viral loads. Subsequently,

mixtures were incubated at 37°C under shaking for 30 min. After incubation, particles were collected by magnetic field and then the remaining plasma were removed.

### **3.8. Measuring HCV RNA in the remaining plasma by Quantitative Real-time RT-PCR**

Isolation of the viral RNA was done using a pathogen free RNA isolation kit from Gene Proof according to the manufacturer's instructions. Real-time RT-PCR was performed according to the manufacturer's protocol for all plasma samples and HCV standards included in the real-time PCR kit. To 40µl master mix, 10µl of extracted HCV RNA was added and the amplification was performed using RotorGeneQ under the following cycling condition: reverse transcription 42°C/5min, initial denaturation 95°C/10 sec followed by 50 cycles of 95°C for 5 sec and 58°C for 40 sec.

### **3.9. Transmission electron microscopy of aptamer-conjugated MNPs and their nanocomplexes with HCV particles**

The aptamer-conjugated MNPs and HCV particles captured by the magnetic nanoconjugates were checked by transmission electron microscopy. For preparing the specimens, 3 µl of 10<sup>-6</sup> dilution of the aptamer-conjugated MNPs and also the captured the virus particles by the nanoconjugates were

immobilized by syringe spraying on agar scientific holey carbon film with 300 mesh Cu(50).

### **3.10. Particle size measurement of MNPs and their nanocomplexes with HCV particles**

The particle size distribution was measured using a Nano-ZS particle sizer based on dynamic light scattering (DLS). For this purpose, 350  $\mu\text{l}$  of  $10^{-6}$  dilution of the MNPs and also the captured virus particles by the nanoconjugates were prepared for measuring by DLS.

#### 4. Conclusions

Chronic hepatitis C is a serious disease that it can result health problems in long-term, including liver damage. It was reported that the viral load suppression was associated with the decreased risk of this liver damage (8).

Current standard-of-care treatment for HCV infection involves the applications of interferons, ribavirin and recently approved protease inhibitors (2). In general, the success of treatment is assessed by reducing concentrations of circulating HCV by extracorporeal methodologies for selectively removing from human samples (14, 20).

Reducing the titer of viral pathogens mechanically as opposed to medicinally may offer several advantages over the current standards of care. For example, our findings suggested that HCV particle concentrations could be mitigated by magnetically filtering a patient's biological fluids using a noninvasive methodology akin to blood dialysis or hemofiltration (21)

In this study, we have demonstrated the aptamer-conjugated magnetic nanoparticles for *in vitro* capturing and isolating HCV viruses from human plasma samples.

Specific conjugate including an aptamer targeted envelope protein of HCV virus, which coated on the surface of magnetic nanoparticles was a useful tool for capturing and concentration of HCV from human plasma samples using a magnetic field. Our finding indicated that the aptamer- conjugated magnetic

nanoparticles had a high affinity for E1E2 glycoprotein on HCV particles and those had also a high efficiency for isolating HCV viruses.

Selective removal via magnetic nanoparticles had previously been demonstrated, for instance for removing metastatic expansion of malignant cells (20) and protamine-adsorbed magnetic nanoparticles for isolation of HCV virus (22). Fluids containing the viruses could be cycled into an extracorporeal circuit where they are selectively captured and removed via a magnetic filtration (21)

In summary, our study provided the specific magnetic nanoconjugate, which efficiently remove HCV particles and decreased the viral load from human plasma samples. The main advantage of this magnetic nanoconjugate was decreasing viral load with noninvasive and non-medically strategy with minimal side effects and could be employed as external therapy for eliminating HCV viruses.

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## Figure legends

**Figure 1.** Schematic formula for bioconjugation of amino-modified E1E2-6 aptamer to starch-coated magnetic nanoparticles

**Figure 2.** Schematic for the binding of HCV viruses with aptamer-conjugated magnetic nanoparticles

**Figure 3.** UV-Vis spectra of magnetic nanoparticles before and after conjugation with amino-modified aptamers. Series 1, UV-Vis spectrum of Apt-E1E2-6; Series 2, UV-Vis spectrum of MNPs with no aptamer; Series 3, UV-Vis spectrum of aptamer-conjugated MNPs

**Figure 4.** FTIR spectra magnetic nanoparticles before (a) and after (b) conjugation with amino-modified aptamers

**Figure 5.** Transmission electron micrographs of magnetic nanoparticles (a), aptamer-conjugated magnetic nanoparticles (b), and aptamer-conjugated magnetic nanoparticles capturing HCV particles in plasma sample (c)

**Figure 6.** Dynamic light scattering of magnetic nanoparticles (series 1) and aptamer-conjugated magnetic nanoparticles capturing HCV particles (series 2) in plasma sample

**Table 1.** Comparison of viral loads in positive samples after challenging by aptamer-conjugated magnetic nanoparticles

	Aptamer-conjugated MNPs (mg/ml)					
	0	$5 \times 10^{-1}$	$5 \times 10^{-2}$	$5 \times 10^{-3}$	$5 \times 10^{-4}$	$5 \times 10^{-5}$
	Viral load (IU/ml)					
Sample 1	11175000	151666	1924333	4204333	7452000	8189666
Capturing Efficiency (%)	0	98.6	83.3	63	28	25
Sample 2	2547000	17000	34666	113000	152000	636666
Capturing Efficiency (%)	0	99.3	98.6	95.5	94	75
Sample 3	406666	115	12000	28333	161666	198666
Capturing Efficiency (%)	0	99.97	97	93	60.2	51.1
Sample 4	2623666	234000	412333	444000	532000	2196666
Capturing Efficiency (%)	0	91	84.2	83	79.7	16.2
	MNPs (mg/ml)					
	0	$5 \times 10^{-1}$	$5 \times 10^{-2}$	$5 \times 10^{-3}$	$5 \times 10^{-4}$	$5 \times 10^{-5}$
	Viral load (IU/ml)					
Sample 1	11175000	11175000	11120666	11117666	11117544	11117632
Capturing Efficiency (%)	0	0	0.5	0.5	0.5	0.5

## Figures

Figure 1.

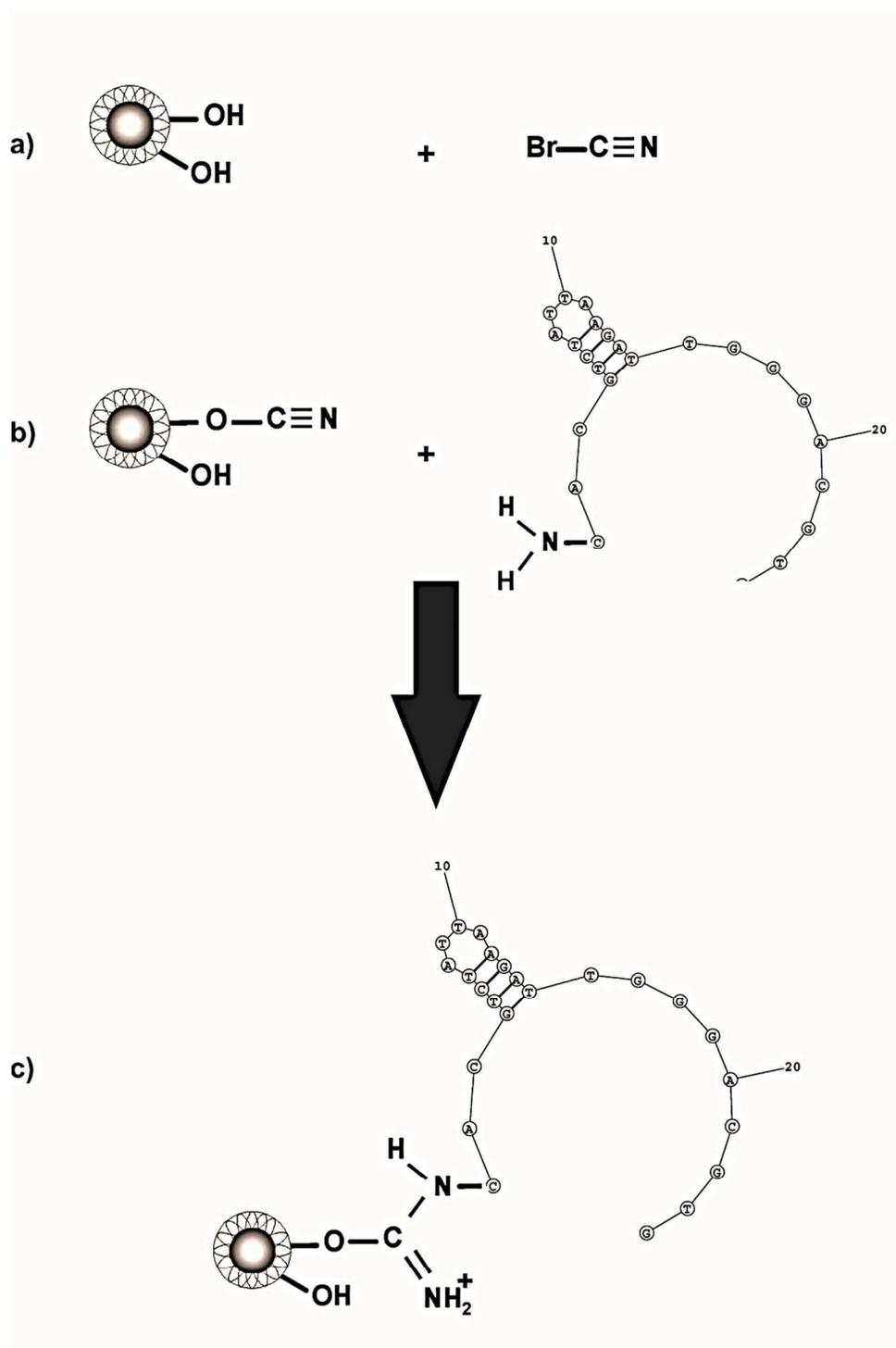


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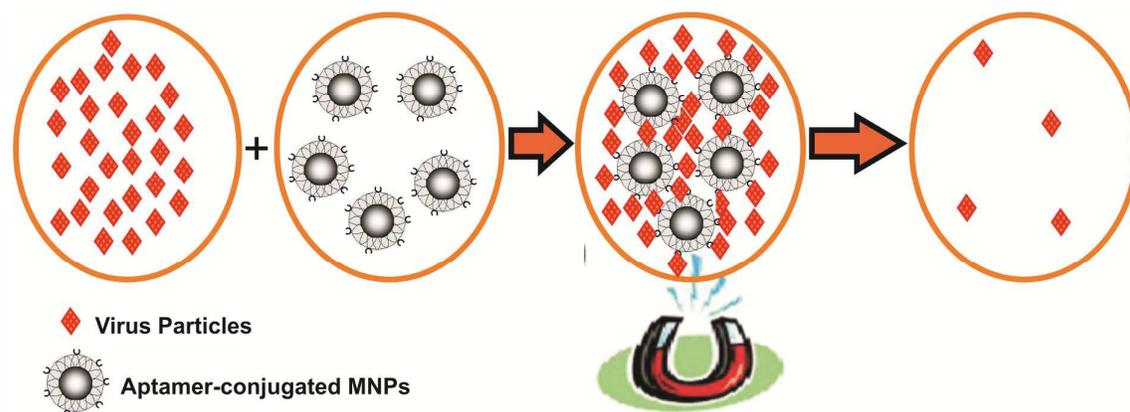


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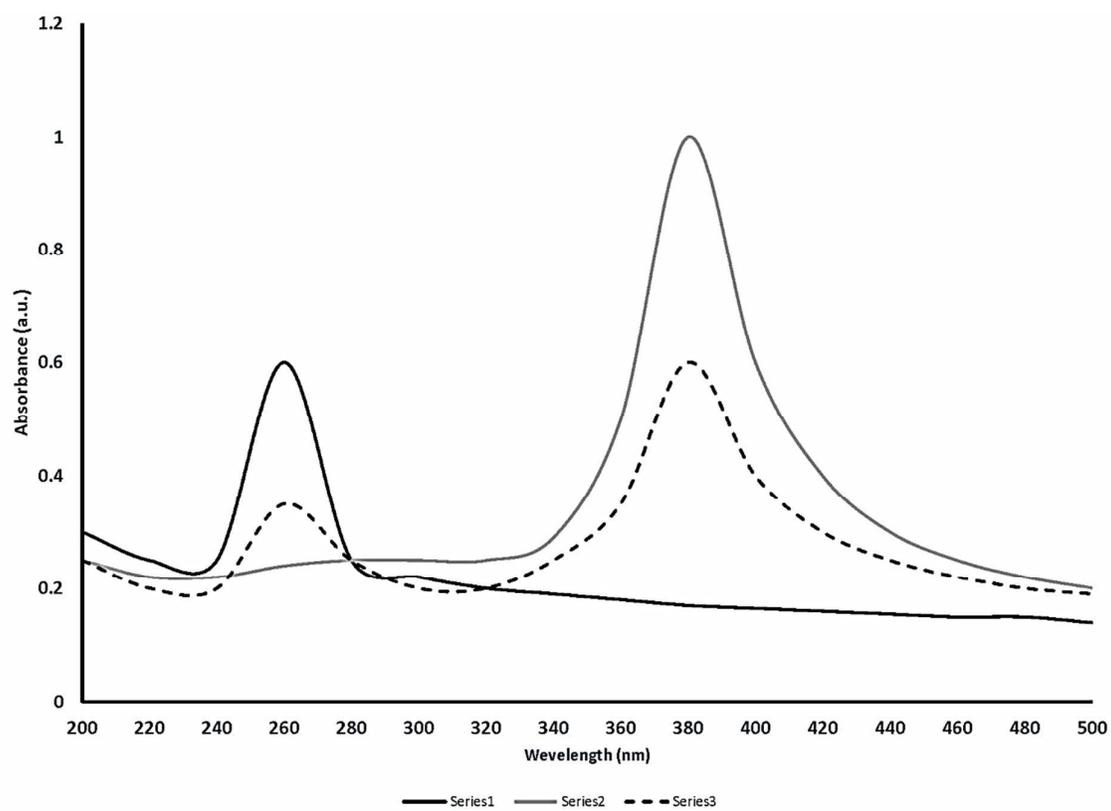


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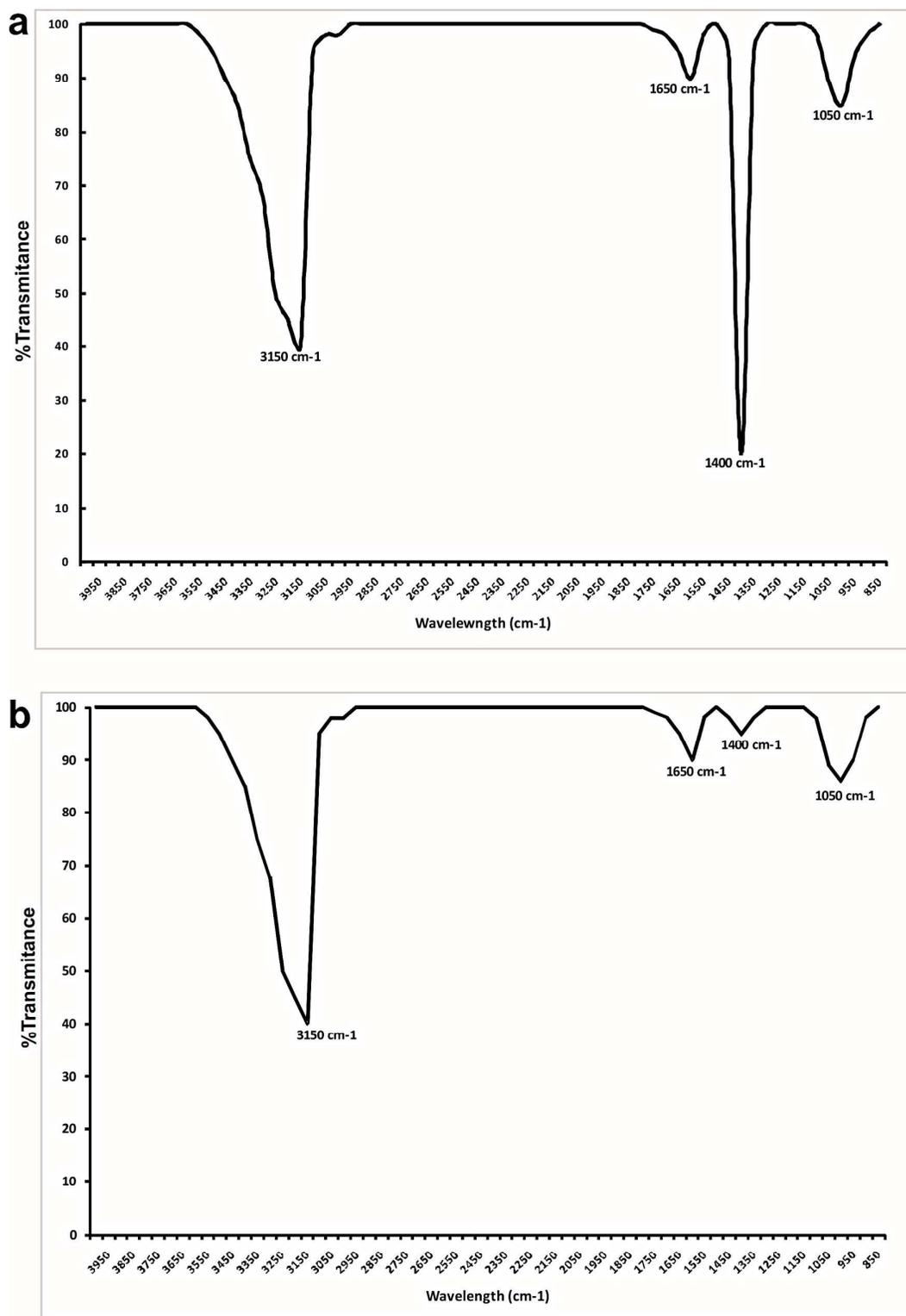


Figure 5.

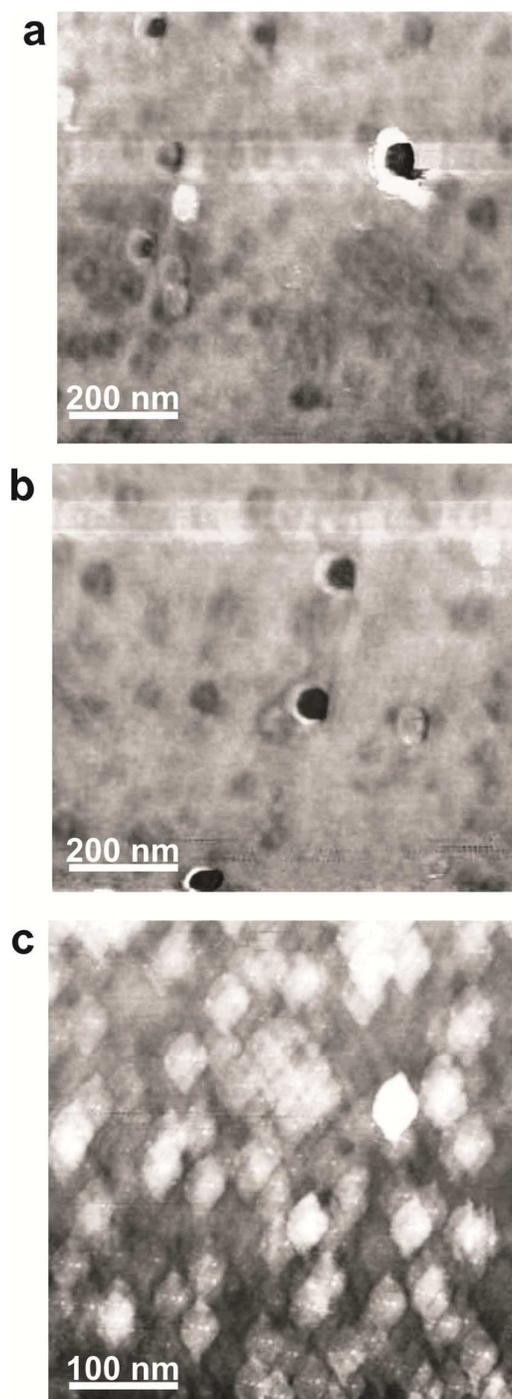


Figure 6

