Evolution of structural and magnetic properties in iron oxide nanoparticles synthesized using Azadirachta indica leaf extract

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Abstract
The novel superparamagnetic nature of magnetic nanoparticles (MNPs) has received significant attention in the wide variety of fields. However, the prerequisites to use these MNPs, particularly in biomedical applications are biocompatibility and high saturation magnetization (\(M_s\)). Thus, the development of a sustainable approach for the synthesis of biocompatible MNPs, which utilizes the redox properties of natural compounds from plant extracts, is highly desired. Herein, we have examined the growth of phase selective MNPs synthesized using Azadirachta indica (Neem) extract as a reducing and capping agent. The physical and biological properties of MNPs synthesized with the modified green hydrothermal method at different reaction times and temperatures were investigated. It was observed that the reaction time and temperature strongly modulated the magnetic and structural characteristics of MNPs. At lower reaction time and temperature of 200 °C for 2 h, primarily iron oxalate hydrate (Fe(C_2O_4).2(H_2O)) was formed. Further, with increasing reaction temperature, the phase transformation from iron oxalate hydrate to pure magnetite (Fe_3O_4) phase was observed. The MNPs prepared with optimum conditions of 220 °C for 4 h show superparamagnetic nature with improved \(M_s\) value of 58 emu g\(^{-1}\). The antibacterial study of MNPs against gram-positive bacteria Staphylococcus aureus showed that the MNPs inhibits the growth of bacteria with the least inhibitory MNPs concentration of 6 μl. Thus, the MNPs obtained by this modified biogenic approach will widen the scope and their applicability in future biomedical applications.

1. Introduction

Superparamagnetic iron oxide nanomaterials are gaining importance in many applications due to their unique magnetic properties. The magnetic storage devices, biosensors, biomedical applications, and magnetic separation technologies are some areas where they have been widely used [1]. Considering the relevance of magnetic nanoparticles (MNPs) in the different applications, numerous physical, chemical, and biological synthesis methods have been developed [2]. The chemical and physical methods are expensive, besides being hazardous. Some of the toxic agents used in chemical synthesis methods like sodium borohydride, hydrogen peroxide, hydrazine, and dimethylhydrazine are harmful, and their minuscule presence put up prejudicial
effects in several applications \cite{3}. To avoid the use of hazardous chemicals, researchers are exploring a green synthesis approach for the preparation of nanoparticles (NPs) \cite{4,5}.

The synthesis methods utilizing biogenic substances such as plant material or plant biomass can be the potential alternative for the chemical and physical methods, as they are cost-effective, and offer low toxicity towards the environment \cite{6}. For the synthesis of metal and metal oxide NPs, the most abundantly available natural resources like plants, bacteria, fungi, yeast, algae, and higher plants part have been used \cite{6–12}. As explained by Adil \textit{et al} the surface capping and reducing ability of phytochemicals present in the extract can be responsible for NPs size control \cite{13}. Natural reductants such as flavonoids, terpenoids, steroids, proteins, organic acid, aldehydes, reductases, dehydrogenases, and extracellular electron shuttles may play a decisive role in the green synthesis of NPs \cite{14}. There are several reports on the green synthesis of iron oxide NPs. Most of these reports include combinational approach, where different extracts such as Artemisia annua \cite{15}, Chinese cabbage \cite{16}, Anana scomosus \cite{17}, Ridge gourd \cite{18}, Banana plantain peels \cite{19} extracts and some leaf extracts like eucalyptus \cite{20}, corn (Zea mays L.) \cite{21}, green tea \cite{22}, Solanum trilobatum \cite{23}, and different Seaweeds \cite{24} are used with the co-precipitation method. Apart from this, some other techniques such as ultra-sonication, hydrothermal, and solvothermal with green approach have also been reported \cite{25–28}. All these reports include the iron oxide NPs with size ranging from 10 to 80 nm.

In previously described methods, MNPs prepared by the green synthesis shows poor magnetic properties and size distribution, which is not desirable. To prepare MNPs with higher saturation magnetization (M_s) along with better control over size, phase, and crystallinity, we have modified a sustainable and environmentally friendly synthesis approach using \textit{Azadirachta indica} extract. \textit{Azadirachta indica}, commonly known as Neem, is a tree native to the Indian subcontinent with antibacterial, antifungal, and anti-inflammatory properties. The phenolic groups and flavones abundantly present in the plant parts of Neem can play an important role in the synthesis of MNPs. The present work reports a green synthesis of Fe_3O_4 MNPs using Neem extract and investigations of their physicochemical and antibacterial properties.

\section*{2. Experimental section}

\subsection*{2.1. Materials}
All the chemicals used are of analytical reagent grade and used without further purification. Ethylene glycol (CH_2(OH)CH_2OH) and Ferric nitrate (Fe(NO_3)_3.9H_2O) were purchased from Sd fine-chem, India.

\subsection*{2.2. Preparation of Neem leaf extract}
The healthy and well matured Neem leaves were collected from the botanical garden of Shivaji University, Kolhapur, India. The collected leaves were washed with double distilled water (DDW) to remove dust and other impurities. To prepare Neem extract, 1 gm Neem leaves were chopped in small pieces and then well grinded in mortar and pestle. Nearly 20 ml of water was used to prepare the paste, which was immediately centrifuged for 20 min at 2500 rpm and filtered with Whatman filter paper No.1. The final volume of centrifuged extract was adjusted to 100 ml with DDW and stored at 4 °C for further use.

\subsection*{2.3. Synthesis of MNPs}
For MNPs synthesis, ferric nitrate was used as an iron precursor. The 0.1 M ferric nitrate solution was prepared in ethylene glycol. Then Neem extract was continuously mixed with ferric nitrate solution in a 1:2 ratio at room temperature and stirred for 30 min. The greenish-black colored solution was obtained after complete addition. Finally, the solution was transferred to Teflon liner, which was sealed and kept it in the furnace at 200 °C for 2 h.
To optimize the temperature of the green synthesis process, the reactions were carried out at 200 °C, 220 °C, and 240 °C for 2 h and samples were named as N1, N2, and N3, respectively. To investigate the effect of reaction time the samples were prepared at 220 °C for 3 and 4 h and named as N4 and N5, respectively.

\subsection*{2.4. Antibacterial activity}
Antibacterial activity of green synthesized MNPs against gram-positive bacteria \textit{Staphylococcus aureus} was determined using a 96 well plate method. For the cultural growth of \textit{staphylococcus aureus}, it was sub-cultured in nutrient agar and incubated at 35 °C for 24 h. For the preparation of the MNPs suspension, 25 mg of MNPs were dispersed in 1 ml sterile water. The broth microdilution test was performed by using sterile, disposable, multi-well microdilution plates (96 U-shaped wells). In all columns, 100 µl media was added as a growth medium. From the second row 10 µl bacterial culture was loaded in all tubes except the first row. For the antibacterial test, the first and second row was kept as a control. 25 µl and 50 µl MNPs suspension were added in the third and fourth row respectively and the \textit{Neem} extract was added in the fifth row. The microtiter plate was covered and incubated at 35 °C for 15 h.
2.5. Characterizations

X-ray diffraction (XRD) measurements were performed on Bruker AXS D2 Phaser diffractometer using Cu-Kα radiation (K = 1.5406 Å). A field emission scanning electron microscope (FE-SEM), Zeiss Ultra 55, was used for the surface morphology and particle size investigations. Micro-structure and particle size were investigated using a transmission electron microscope (TEM), Tecnai G2 S-Twin, at voltage 200 kV. Fourier transform infrared (FTIR) spectra were recorded at room temperature by in the range of 400–4000 cm⁻¹ by a Shimadzu FTIR spectrophotometer. The dynamic light scattering (DLS) measurements were performed with ZETA-PSA ELS-8000 at 300 K. Magnetic measurements were carried out using Quantum Design’s VersaLab Physical Property Measurement System. Magnetization versus field (M-H) loops for all the samples were measured at 300 K and 60 K in magnetic fields up to 30 kOe. Zero field-cooled (ZFC) and field-cooled (FC) magnetization measurements were performed in the temperature range 50–300 K in an applied magnetic field of 500 Oe. The elemental analysis of samples was obtained by x-ray photoelectron spectroscopy (XPS) (Thermo VG Scientific, U.K.) with a monochromatic Mg-Kα (1253.6 eV) radiation source.

3. Results and discussion

3.1. Effect of reaction temperature

The X-ray diffraction patterns of samples N1, N2, and N3 prepared at temperatures 200 °C, 220 °C, and 240 °C, respectively, are shown in figure 1. The diffraction patterns show the growth of crystalline MNPs in different orientations for the respective lattice planes. The sample N1 prepared at 200 °C shows the diffraction patterns for the iron oxy-hydroxide phase, i.e., iron oxalate hydrate (JCPDS-01-088-0315) [29]. The intense diffractions peaks at 2θ values of 18.53°, 23.10°, 24.42° and 29.64° for corresponding (h, k, l) planes (−2 0 2), (0 0 2), (−1 1 2) and (−4 0 2) respectively are indicated by ‘*’ in figure 1. The sample N2 and N3 showed characteristic diffractions peaks for the planes (2 2 0), (3 1 1), (4 0 0), (5 1 1) and (4 4 0) corresponding to pure Fe₃O₄ phase with a cubic structure (JCPDS-01-088-0315) [25]. The phase formation of MNPs was observed to be strongly influenced by the reaction temperature. In the previous study, it was observed that there is a possibility of the formation of zero-valent iron NPs at the first stage and these iron NPs could then be transferred to the iron oxide phase after solvothermal treatment [25]. Sample N1 has mixed iron oxy-hydroxide phases of iron oxide, indicating the 200 °C is not adequate temperature for the formation of the magnetite phase. The boiling point of ethylene glycol is 197 °C and the reduction process may remain incomplete at 200 °C. The nucleates formed at the initial stage of the reaction can create an easy path for grain growth; however, the elevated temperature over the boiling point of the solvent is required for the growth of MNPs [30]. Hence, the samples N2 and N3 synthesized at a higher temperature show the formation of the Fe₃O₄ phase compared to N1.

Figures 2(a)–(c) shows the FE-SEM images, and figures 2(d)–(f) shows the TEM images of samples N1, N2, and N3, respectively. Samples N1, N2, and N3 were nearly spherical but are observed in the aggregated state. With increasing reaction temperature, well-shaped NPs formation was observed. The particle size measured for
samples N1, N2, and N3 from TEM images are in the range of 3–6, 8–11, and 7–14 nm, respectively. The histogram obtained from HR-TEM images for sample N2 and N3 (figure S1 is available online at stacks.iop.org/NANOX/1/020013/mmedia) shows the average particle size of 8 and 9 nm, respectively. As samples N1 have low crystallinity, it is a bit difficult to determine the precise size. The sample N1 and N3 are in the highly agglomerated state, whereas sample N2 has a nearly homogeneous distribution of MNPs with lower aggregation. The SAED patterns shown in figures 2(g)–(i) for samples N1, N2, and N3 respectively corroborate well with XRD results. From these structural and microstructural investigations, it is evident that the reaction temperature used for preparing the N2 sample (220 °C) is the optimum temperature for Fe3O4 phase formation. This temperature was used to investigate the effect of reaction time on the structural and morphological properties of MNPs.

3.2. Effect of reaction time
The reaction time was varied to improve crystallinity and grain growth. For this, the reaction temperature was fixed at 220 °C, and the reactions were carried for 3 h and 4 h, and corresponding samples were labeled as N4 and N5, respectively. Figure 3(a) shows the FE-SEM image of sample N4 and it can be seen that MNPs have a nearly spherical shape. The TEM micrographs of different magnifications are shown in figures 3(b), (c). These micrographs display MNPs with a spherical shape, lower aggregation, and nearly homogeneous size distribution. For sample N4, the particle size measured from the TEM micrograph was found to be around 8–14 nm. The corresponding histogram drawn for sample N4 shows that the average particle size is 11 nm (figure S1). The diffraction rings in the SAED pattern depicted in the inset of figure 3(b) are indexed with the Fe3O4 cubic phase. The high crystallinity and nearly spherical shape (hexagon) of single MNP are evident in figure 3(c). All the rings in the SAED pattern are intense and are consistent with the XRD pattern shown in

![Figure 2. FE-SEM images (a)–(c), TEM images (d)–(f), and SEAD patterns (g)–(i) of samples N1, N2, and N3, respectively.](image-url)
It signifies that the development of crystalline MNPs can occur when the reaction time was maintained up to 3 h.

For sample N5, the FE-SEM image is shown in figure 4(a), and TEM micrographs are shown in figures 4(b), (c). It was observed that along with spherical morphology, N5 particles have a low degree of aggregation than sample N4. The particle size measured for sample N5 from the TEM micrograph was found to be around 11–16 nm. Also, the average particle size determined from the TEM size histogram was about 13 nm (figure S1), with a standard deviation of 3.7 nm. The DLS measurement performed for optimized sample N5 reveals the average hydrodynamic size around 340 nm with a polydispersity of 0.205 (figure S2). The high-resolution TEM image, as shown in figure 4(c), displays the high crystallinity and spherical shape of single MNP. The SAED pattern for N5 is shown in the inset of figure 4(b), which exhibits diffraction rings corresponding to the cubic phase of Fe3O4, which is consistent with the XRD pattern shown in figure 4(d).

3.3. FT-IR analysis

The FT-IR spectroscopy was used to investigate the functional groups present on the surface of green synthesized MNPs. Figure 5 shows the FTIR spectra of Neem leaf extract and synthesized iron oxide NPs. The strong band observed at 491 cm⁻¹ (for samples N1), and 555 cm⁻¹ (for samples N2 to N5) was ascribed to the Fe–O bond of iron oxide. The shift in the position of the Fe–O bond was observed with the formation of the pure Fe3O4 phase [31]. The peak at 781 cm⁻¹ was assigned to the stretching vibration of aromatic alkanes (C–H) [14]. For all NPs samples, a sharp peak observed at 1315 cm⁻¹ is due to the presence of an aromatic amine (C–N) bond [20]. A peak around 1621 cm⁻¹ corresponding to (C=O) bond is due to the existence of proteins such as flavanones/terpenoids from plant extract [28, 32]. These proteins may get involved in the reduction of metal ions. In all the spectra stretching vibrations observed in the range of 3300 cm⁻¹ to 3350 cm⁻¹ were allocated to the free (–OH) bonds from polyphenols besides water [33]. The plant extract spectra also exhibit stretching and bending modes for N–H and NH3 groups corresponding to phenolic groups present in the extract [34]. From the analysis of FT-IR spectra, we can confirm that several carboxyl and polyphenolic groups are present in all the samples. The biomolecules like terpenoids and polyphenolic flavonoids are abundant in Azadirachta indica plant.

Figure 3. (a) FE-SEM image (b) TEM image with the SAED pattern in the inset (c) HR-TEM image, and (d) XRD pattern of sample N4.
Figure 4. (a) FE-SEM image (b) TEM image with the SAED pattern in the inset (c) HR-TEM image, and (d) XRD pattern of sample N5.

Figure 5. FTIR spectra of samples N1, N2, N3, N4, N5, and Neem leaf extract.
extract and hence observed on the surface of green synthesized MNPs. The occupancy of these biomolecules from extract around MNPs surface confirms the capping activity during the growth of the MNPs.

3.4. XPS analysis
The high-resolution XPS analysis of samples N1 and N5 was carried out to investigate the phase and oxidation states. XPS investigations are useful to differentiate between various phases of iron oxides, as it is very sensitive to Fe$^{2+}$ and Fe$^{3+}$ cations. In the survey spectra of sample N1, the small hump for the N 1 S (nitrogen) was observed (figure S3). The presence of nitrogen to a smaller extent confirms the presence of amine groups on the MNPs surface, supporting the FTIR data. In the Fe spectra of N1 and N5 samples (figures 6(a) and (c)), the characteristic doublet for core electrons of Fe peaks positioned at 711.1 eV and 724.1 eV corresponding to Fe$^{2+}$/2 and Fe$^{3+}$/2 orbitals respectively, were observed [35, 36]. For sample N1 the additional satellite peak for Fe$^{3+}$ around 719.0 eV validates the formation of iron oxy-hydroxide or the presence of excess oxide and hydroxide in the sample [37]. The absence of an additional satellite peak in Fe spectra of sample N5 confirms the phase purity and formation of the pure magnetite phase [38]. In the oxygen spectra of both samples (figures 6(b) and (d)), the characteristic peak at 530.3 eV corresponding to O 1 s orbital was observed [39]. The similar binding energy was reported by Cuenca et al [37]. The deconvoluted oxygen spectra for both N1 and N5 samples (figures 6(b) and (d)) shows three peaks at 529.3 eV, 531.7 eV, and 533.4 eV corresponding to the (Fe–O), carboxyl (C–O) and hydroxyls (O–H) bonds respectively. In the N1 sample, the presence of impure phase and organic compounds, i.e., C, H, and O elements, leads to broader spectra compared to that of N5, signifying sample N5 have a pure phase of Fe$_3$O$_4$ [39].

3.5. Magnetic properties
The room temperature (300 K) and low temperature (60 K) magnetization curves of all the samples are shown in figures 7(a) and (b) respectively. All the magnetization curves are not saturating even at an applied field of
Also, the room-temperature magnetization curves did not show any coercivity. This non-saturating and non-coercive behavior at room temperature point out the superparamagnetism in our bio-synthesized MNPs [40]. For samples, N1 to N5 $M_s$ values have been increased from 13 emu g$^{-1}$ to 58 emu g$^{-1}$ at 300 K and 19 emu g$^{-1}$ to 66 emu g$^{-1}$ at 60 K. The $M_s$ value for all the samples is less than that of bulk Fe$_3$O$_4$. This reduced $M_s$ values can be attributed to the surface spin disorder at the nanoscale and nonmagnetic surface coatings as observed from FTIR [25, 41]. There are some reports which also achieved higher $M_s$ by the green approach [42, 43]. The magnetic curves measured at 60 K have non-zero coercivity and higher $M_s$ values than at 300 K.

Temperature-dependent magnetization behaviour was analyzed through zero-field cooling (ZFC) and field cooling (FC) measurements as shown in figure 8. The black solid circles and white open circles represent the FC and ZFC data, respectively. The FC-ZFC curves of sample N1 show reversible behaviour in the measured temperature range. On the other hand, for samples N2–N5 the FC and ZFC curves are irreversible and exhibit a broad peak in the ZFC curve indicating their blocking temperature ($T_B$) in the range of 100 K–200 K. The broadening in the ZFC curve is observed due to the size distribution of the MNPs. In the TEM size histogram (figure S1), N2 shows the narrower size distribution compare to N3 and N4, while the ZFC-FC results show the broader size distribution of N2. TEM histogram represents the size distribution of NPs, which have the magnetic

Figure 7. (a) The room temperature (300 K) and (b) low temperature (60 K) magnetization curves of samples N1–N5.

Figure 8. Temperature-dependent magnetization curves (ZFC-FC) from (a) to (e) measured at $H = 500$ Oe for sample N1 to N5, respectively.
core and nonmagnetic biomolecules from extract on the surface. On the other hand, ZFC-FC results reflect the size distribution of magnetic core only. The samples N3 and N4 were prepared with either higher temperature or higher time compared to N2 and have a better grown magnetic core. Therefore even though N2 has a narrower TEM size histogram, ZFC-FC results show narrower size distribution for N3 and N4 compared to N2. The detailed magnetization data for all the samples are tabulated in table 1.

The $M_s$ values are increased with increasing temperature and reaction time. Ozel et al have observed similar improvements in magnetic properties with increasing time and temperature [41]. This improvement in $M_s$ values can be attributed to an increase in MNPs size and crystallinity at optimum conditions [44]. In the samples investigated, the highest $M_s$ value of 58 emu g$^{-1}$ at room temperature was obtained for sample N5. The comparison of $M_s$ values of MNPs obtained by this modified green synthesis approach with previously reported literature is presented in table 2.

### Table 1. Magnetization data of samples N1 to N5 extracted from magnetic measurements.

| Sr No. | Sample code | Reaction condition | Average size (nm) | Blocking Temperature $T_B$ (K) | $H_C$ (Oe) | $M_s$ (emu g$^{-1}$) | $H_C$ (Oe) | $M_s$ (emu g$^{-1}$) |
|--------|-------------|--------------------|-------------------|-------------------------------|------------|---------------------|------------|---------------------|
| 1      | N1          | 200 °C (2 h)       | —                 | —                             | 13         | 12                  | 12         | 19                  |
| 2      | N2          | 220 °C (2 h)       | 8                 | 160                           | —          | 47                  | 79         | 54                  |
| 3      | N3          | 240 °C (2 h)       | 9                 | 110                           | —          | 49                  | 40         | 57                  |
| 4      | N4          | 220 °C (3 h)       | 11                | 120                           | —          | 54                  | 58         | 61                  |
| 5      | N5          | 220 °C (4 h)       | 13                | 160                           | —          | 58                  | 52         | 66                  |

### Table 2. Comparison of $M_s$ values of green synthesized MNPs from the literature.

| Sr No | Synthesis Method (extract) | $M_s$ (emu g$^{-1}$) | References |
|-------|----------------------------|----------------------|------------|
| 1     | Watermelon rind extract   | 14.2                 | Prasad et al [45] |
| 2     | Punica Granatum rind extract | 22.7            | Venkateswarlu et al [46] |
| 3     | Green tea extract         | 16–20                | Xiao et al [47] |
| 4     | Green tea extract         | 49                   | Karade et al [48] |
| 5     | Neem extract              | 58                   | Present work |

Figure 9. (a) Antibacterial activity of MNPs with 25 and 50 μl concentrations and extract determined by 96 well methods (optical absorbance) and (b) Minimum inhibitory concentration of MNPs from 2 μl–10 μl.

3.6. Antibacterial activity

The antibacterial activity of extract and MNPs with different concentrations was determined through 96 well method. After incubation, the absorbance values were recorded for the growth of each segment and are shown in figure 9(a). From this assay, it was revealed that the green synthesized MNPs show higher inhibition activity towards bacteria, pointedly higher than Neem extract at the lower concentration of MNPs. The MNPs with 25 μl concentration shows higher antibacterial or inhibition activity compared to 50 μl MNPs. It signifies that the size
and density of MNPs play an important role in inhibiting the growth of bacteria, as the size of NPs has functional activities that help to destruct bacterial growth [49]. The mechanism behind the antibacterial activity has not been fully understood and explained yet. However, Kim et al described the relationship between the oxygen-free radicals from NPs and antibacterial activity [50]. Higher the active surface area of NPs, better they can get adsorbed over the bacterial cell surface, which subsequently can damage the cell wall of bacteria. The exposure of intracellular components of the bacterial cell to NPs leads to the leakage of them, prohibiting the rapid growth of bacteria [51]. For further detailed investigation and confirmation of activity from MNPs, the minimum inhibitory concentration (MIC) was estimated by the microdilution process. In the MIC assay, the concentrations (2 μl–10 μl), which were used, are less than the active concentration (25 μl) against bacterial culture. All concentrations show antibacterial activity against bacteria (figure 9(b)). At extremely low and high concentrations of MNPs, i.e., 2 μl and 10 μl, the lower inhibitory activity against bacteria was observed. The extremely low and high concentration of MNPs may offer lower surface exposure to the bacteria showing minimal activity. On the contrary, the highest inhibitory activity against bacteria was observed at an optimum concentration of 6 μl.

4. Conclusions

Recognizing the need for the development of sustainable, green, and eco-friendly synthesis methods, the MNPs were synthesized using Azadirachta indica (Neem) aqueous plant extract. The key findings of the study are summarized below.

(a) The combinational approach of the modified green and hydrothermal method gave control over the size and morphology of MNPs.

(b) The reaction temperature significantly affected the phase of MNPs. An increase in reaction temperature from 200 °C to 220 °C has resulted in the transfer of iron oxyhydroxide phase to the pure magnetite phase.

(c) At optimum reaction conditions (220 °C for 4 h) spherical MNPs of the size range, 13–15 nm with good crystallinity was obtained.

(d) Polyphenols present in the extract acts as the capping and reducing agent for ferric ions during the synthesis of MNPs. The XPS and FT-IR analysis displayed the existence of O-H, C-H, and C=H bonds from polyphenols on MNPs surface.

(e) The MNPs have superparamagnetic nature at room temperature. The MNPs with optimum conditions exhibits the highest M_s value of 58 emu g⁻¹.

(f) The antibacterial activity revealed that MNPs successfully inhibited the growth of gram-positive bacteria Staphylococcus aureus with a minimum inhibitory concentration of 6 μl for MNPs.

(g) The MNPs prepared by this modified green approach can be effectively used in biomedical applications such as MRI, drug delivery, and hyperthermia applications.

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Associated content

Supporting Information.

Conflicts of interest

There are no conflicts of interest to declare.
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