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Design of enzyme immobilized polymer-brush-grafted magnetic nanoparticles for efficient nematocidal activity

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Parasitic nematodes are not only causing deadly diseases in plant and animals but also adversely affecting agriculture industry and global health, particularly in developing countries. In this work, we planned to hyphenate the concept of enzyme immobilization with nanotechnology to develop magnetic nanoparticles (MNPs) with efficient nematocidal activity in water. A novel nematocidal platform is developed by immobilizing protease (from *Streptomyces grisues*) on the surface of polymer brush grafted magnetic nanoparticles (MNPs-PGMA-Pro). For comparison, a monolayer based nematocidal platform is also developed by functionalizing protease on the surface of glutaraldehyde functionalized MNPs (MNPs-GA-Pro). MNPs-PGMA-Pro particles show enhanced enzyme activity and stability over a wide range of temperature and pHs as compared to MNPs-GA-Pro. Polymer brush and monolayer based protease functionalized MNPs exhibit superior enzyme activity when compared to the free enzyme. When tested for nematocidal activity against the parasitic nematodes (*Haemonchus contortus*), the polymer brush based platform retains a higher activity over 7 cycles of magnetic separation. The reported platforms can be prospectively employed for the water treatment while the reusability over many remediation cycles due to the facile magnetic separation promises a substantially reduced treatment cost.

1. Introduction

Recently, nanotechnology has attracted remarkable scientific interest for its applications in almost all the facets of everyday lives. Among nanoparticles, Fe_3O_4 magnetic nanoparticles (MNPs) are widely employed as carriers of bioactive molecules such as enzymes and drugs, and present interesting attributes of high surface area, biocompatibility, convenient magnetic separation and drug delivery.[1, 2] Various methods have been developed for the immobilization of enzymes on the surface of MNPs such as adsorption or via covalent conjugation to carboxylic acid, aldehyde, thiol, epoxide, and maleimide group.[3, 4] Polymer brushes, defined as surface tethered polymeric chains, are excellent candidates for modulating surface chemical and physical properties of a wide variety of materials. The key advantages associated with the polymer brushes when compared to other surface functionalization strategies include higher stability and activity inherent with their covalent nature and higher density of active groups on the surface.

Bio-inspired materials and bioactive natural products are widely explored for biocatalytic applications due to their inherent activity and biocompatibility. Protease enzymes are well recognized for their proteolytic activities and are employed for proteomic analysis, hydrolysis of proteins in variety of substrates such as wool, rapeseed, and enzymatic degradation of synthetic polymers.[3, 5-7] Protease is reported to be secreted by some fungal and bacterial species as biological control against nematodes where nematocidal activity is achieved by destroying the cuticle of the target.[8] Thus naturally isolated protease enzymes are attractive candidates as nematocides. The stability and activity of enzymes are fundamentally affected by various physiological and environmental parameters. Immobilization of enzyme to the surface is an effective strategy for enhancing stability, activity and in some cases reusability of enzyme.[9-11]

Parasitic nematodes are not only known for their harmful impact on plant and animal but are also recognized for causing many deadly diseases in humans.[12] Their free-living species are

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abundant in fresh and salt water bodies and soil habitats. Higher concentration of nematodes in drinking water causes to add unpleasant taste to the drinking water and these nematodes may carry pathogenic bacteria in their gut.[13] In this work we tested *Haemonchus contortus* as a sample nematode in drinking water because it is a blood-feeding nematode effecting ruminants, responsible for anaemia and death of grazing animals, thereby causing serious impact on livestock industry.[14, 15]

Considering these problems, in the present work extracellular protease enzyme was covalently immobilized on the surface of polymer brush functionalized MNPs. Poly (glycidyl methacrylate) (PGMA) brushes were grafted on the surface of the MNPs particles by surface initiated atom transfer radical polymerization (SI-ATRP), followed by covalently binding the enzyme. For comparison, a monolayer based platform was also developed by immobilizing protease to the surface of gluteraldehyde modified MNPs. The polymer brush based platform exhibited superior nematocidal activity and stability of the enzyme over wide range of pH and temperature as compared to the monolayer based platform and free protease.

Scheme 1. Schematic illustration of protease immobilization to the surface of monolayer and polymer brushes functionalized magnetic nanoparticles.

2. Experimental section

2.1. Materials

Peptone (98%), yeast extract (99%), NaCl (98%), Casien (99.9%), Glucose (98%), Soyabean meal (99%), KH_2PO_4 (99%), $MgSO_4$ (99%), Na_2CO_3 (99%), Trichloroacetic acid (TCA) (99%), Alkaline reagent (99%), Folin reagent (99%), $FeSO_4$ (>99%), KNO_3 (99%), KOH (>90%), Ethanol (>99%), NaH_2PO_4 (97%), Na_2HPO_4 (98%), Ammonia solution (35%), (3-Aminopropyl) triethoxysilane (APTES) (99%), Glutaraldehyde (50% in water), and Tetraethylorthosilicate (TEOS) (99%), α-bromoisobutyryl bromide (99%), Glycidyl methacrylate (GMA) (99%), bipyridyl, $Cu(II)Br_2$ (99%), Cu(I)Br (99%) were purchased from Sigma Aldrich, Germany. All reagents were used without further purification. Protease (PRONASE® Protease, *Streptomyces griseus*) was purchased from Merck KGaA, Germany.

2.2. Immobilization of protease on magnetic nanoparticles (MNPs-GA-Pro)

$FeSO_4.7H_2O$ (20 g, 72 mmol) was dissolved in 140 mL of deionized water, pre-degassed with nitrogen, and the temperature was adjusted at 90°C. Solution of KNO_3 (1.62 g, 16 mmol) and KOH (11.23 g, 200 mmol) was prepared in 60 mL of H_2O. This solution was added dropwise to the above solution in approximately 5 min under nitrogen bubbling. The reaction mixture was heated at 90°C under mechanical stirring for 1 h. The solution was left overnight at room temperature. MNPs were separated magnetically and washed several time with de-ionized water. For coating silica, MNPs (200 mg) were dispersed in 150 mL of ethanol and solution was kept under sonication for 15 min while immersed in ice. Ammonia solution (12 mL, 35%) and TEOS (400 µL, 1.8 mmol) were added to the suspension and left in ice bath for sonication for 2 h. The particles (MNPs-OH) were separated from reaction mixture by using permanent magnet and washed thoroughly with ethanol. For the preparation of amine functionalized nanoparticles, APTES (13 mL, 55.5 mmol) was added in ethanol (320 mL) followed by addition of MNPs-OH particles (1 g). The mixture was kept under mechanical stirring for 3 h at 40°C. The black particles ($MNPs-NH_2$) were separated from reaction mixture and washed thoroughly with ethanol.

$MNPs-NH_2$ particles (200 mg) were disperse in 5% solution of glutaraldehyde (GA) in 0.1 M phosphate buffer solution (25 mL, pH = 7). The mixture was stirred at room temperature for 6 h. The nanoparticles (MNPs-GA) were washed thoroughly with phosphate buffer and separated by magnet.

Immobilization of proteases on MNPs-GA particles was carried out by using proteases enzyme (20 mg) in phosphate buffer solution (20 mL, pH = 7). MNPs-GA particles (110 mg) were dispersed in the above solution and placed on shaker for 12 h at room temperature. Treated particles were separated from reaction mixture by using permanent magnet and washed with de-ionized water.

2.3. Immobilization of enzyme on polymer-brush-grafted magnetic nanoparticles (MNPs-PGMA-Pro)

Briefly, solution of TEA (120 µL, 0.86 mmol) and α-bromoisobutyryl bromide (90 µL, 0.72 mmol) in DCM (7 mL) was degassed for 15 min at r.t. and injected over degassed $MNPs-NH_2$ (330 mg) particles under inert atmosphere. The reaction mixture was mechanically stirred under inert atmosphere for 2.5 h at r.t. The particles (MNPs-BI) were

separated by permanent magnet and washed twice each with ethanol and DCM.

GMA (5.7 mL, 42.9 mmol) was dissolved in DMF:H2O (12 mL, 2:1), followed by addition of bipyridyl (83 mg, 0.532 mmol) and $Cu(II)Br_2$ (11.7 mg, 0.052 mmol). After degassing for 1 h, Cu(I)Br (40 mg, 0.28 mmol) was added it and solution was degassed for another 15 min. The above solution was injected over MNPs-BI under inert environment. The polymerization was carried out for 12 h at r.t. and subsequently the particles (MNPs-PGMA) were washed 3 times each with acetone and THF.

Proteases was immobilized on polymer-brush-grafted fabricated MNPs by dispersing MNPs-PGMA (110 mg) in solution of proteases enzyme (20 mg) in PBS (20 mL, pH = 7). The suspension was shacked for 12 h at room temperature and particles were separated by using permanent magnet followed by repeated washings with de-ionized water.

2.3. Characterization

Scanning electron microscopic (SEM) imaging was performed on Hitachi SU8000 SEM. Transmission electron microscopic (TEM) imaging was carried out on Philips EM 420 and FEI Tecnai G2 F30 instruments. A Physical Property Measurement System (PPMS) device (Cryogenic, VSM 9 T Magnet) at 295K was used to measure magnetic properties of MNPs. ATR-FTIR spectra were recorded on ATR-FTIR Alpha Bruker spectrometer. Thermogravimetric analysis (TGA) was performed with a TA Instruments device (Model TGA Q500) from r.t. to 1000°C at a heating rate of 10°C/min under a nitrogen gas (N_2) atmosphere (40 mL/min). Other instruments include Incubator (EHRET- BK4444, Germany), Spectrophotometer (Nicolet 300 Thermo, USA), Centrifuge (Sigma 2-6, USA). The zeta potential and hydrodynamic radii of the MNPs in phosphate buffered saline (PBS) were measured with Malvern ZetaNano ZS at 25°C. A U-shaped capillary cell DTS1070 was used to estimate the zeta potential using a He-Ne laser source of 5 mW at 633. Each sample was measured three times, and the values reported (Table SI-2) are average of these measurements.

2.4. Enzyme essay

Assay of free and immobilized protease was done by McDonald and Chen method.[16] Casein (4 mL) was added in suitable amount of enzyme and incubated at 37°C for 30 min. 1% Trichloroacetic acid (1 mL) was added to stop the reaction. The reaction mixture was centrifuged at 6000 rpm for 10 min. Alkaline reagent (5 mL), 1N aq. NaOH (1 mL) and folin reagent (1 mL) were added in supernatant (1 mL). The absorbance of blue color complex was measured with UV/Vis spectrophotometer at 700 nm. One unit of protease activity (U) is defined as the amount of enzyme required to cause an increase of 0.1 in optical density at 700 nm under defined conditions. Effects of physical parameters on enzyme activity was determined by taking samples from reaction mixture after 0, 12, 24, 36 and 48 h under different experimental conditions (temperature and pH).

2.5. Effect of temperature and pH on protease activity

The effect of temperature on the activity of free and immobilized proteases enzymes were studied. Both free and immobilized proteases were incubated at different temperature ranging from 25 to 80°C. The amount of free enzyme used was 1 mg in each case while the amount of MNPs-PGMA-Pro employed was 5.5 mg. The 5.5 mg amount of MNPs-PGMA-Pro was chosen because at this amount the nematocidal activity of MNPs-PGMA-Pro was comparable to the nematocidal activity of 1 mg of free enzyme. The activity was measured by performing standard assay method. Similarly, the effect of medium pH on the activity of enzyme was investigated by incubating free (1 mg) and immobilized protease (5.5 mg) in PBS solution of different pH ranging 5-12. The activity of enzyme was measured by incubating casein and proteases enzyme in PBS of different pH at 37°C. The activity was measured by performing standard assay method.

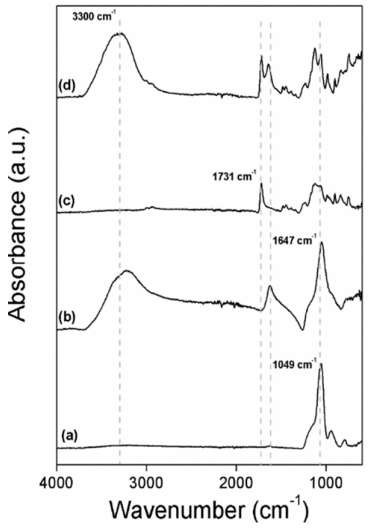

Figure 1. ATR FTIR Spectra MNPs-NH2 (a), MNPs-GA-Pro (b), MNPs-PGMA (c), MNPs-PGMA-Pro (d).

2.6. Nematocidal activity of immobilized proteases (MNPs-GA-Pro and MNPS-PGMA-Pro)

Bioactivity of free and immobilized proteases was determined by using standard assay method with regular intervals to check the effect of particles on nematodes. Nematodes were washed thoroughly with PBS (pH = 7.0) before use. 30 µL of nematode stock solution (contains approximately 50-60 nematodes) was transferred into 1.5 mL Eppendorf tube. Functionalized MNPs (MNPs-GA-Pro and MNPs-PGMA-Pro) (1 mg) were added in it and the mixture was incubated at 37°C for 12, 24, 36 and 48 h. As a control, blank samples were run parallel to immobilized proteases under same conditions. The effect of immobilization on the efficiency of protease was investigated by observing numbers of living and dead nematodes under light microscope and later confirmed by using scanning electron microscope.

3. Results and discussion

MNPs particles with an average size of ~100 nm were prepared by the simple alkaline hydrolysis of FeSO4. MNPs were suspended in ethanol and treated with TEOS under basic conditions. This treatment resulted in the silica coated NMPs. Silica coating of MNPs was performed to facilitate the subsequent silanization process employing APTES that resulted in the amine functionalized MNPs (MNPs-NH_2).[17] The surface amino groups of MNPs-NH_2 were reacted to the glutaraldehyde (GA) under ambient condition resulting in MNPs with surface aldehyde groups (MNPs-GA).[18] The surface aldehyde groups of MNPs-GA were conjugated to the amino groups of protease via imine linkages resulting in the monolayer based protease functionalized MNPs platform (MNPs-GA-Pro). For fabrication of protease grafted polymer brushes based MNPs platform, PGMA brushes were grown from the surface of ATRP initiator functionalized MNPs (MNPs-BI) by SI-ATRP in the presence of Cu(I)/Cu(II)-BiPy catalyst system.[19] The complete MNPs functionalization strategy is illustrated in Scheme 1.

All the surface functionalization steps were monitored by ATR-FTIR spectroscopy. MNPs show typical absorption band at 546 cm^{-1} corresponding to Fe–O stretching vibration. The silica coating of MNPs after the treatment with TEOS was established by the characteristic absorption band for Si–O–Si stretching vibration at 1049 cm^{-1}.[20] In case of monolayer based platform, the immobilization of protease on MNPs-GA was supported by the adsorption band at 1639 cm^{-1} due to the stretching vibration of carbonyl groups in protease. For the polymer brush based platform, the successful polymer brush growth for MNPs-PGMA was confirmed by the absorption band at 1731 cm^{-1} originating from the stretching vibration of carbonyl (C=O) groups in PGMA. The subsequent conjugation of protease via a reaction with the epoxide group of PGMA was supported by the absorption bands at 1631 cm^{-1} and 3300 cm^{-1} due to amide and N-H bond stretching, respectively (Figure-1).[19]

The successful chemical modification of MNPs surface were further corroborated by XPS analysis (Figure 2). The magnetite (Fe3O4) nature of MNPs used in this study was confirmed by the binding energy signals of Fe2p1/2 and Fe2p3/2 at 725 eV and 711 eV (Figure SI-1a). The APTES functionalization of MNPs resulted in the appearance of characteristic signals for silicon at 153 eV and 103 eV for Si2s and Si2p respectively. Additionally, the peak at 400 eV for N1s substantiated the successful functionalization on MNPs surfaces with APTES (Figure SI-1b).[20] The conjugation of surface amino groups in MNPs-NH_2 with the glutaraldehyde brings more carbon content at the surface of MNPs, which was substantiated by the decrease in N/C ratio from 0.079 in MNPs-NH_2 to 0.046 in MNPs-GA. An increase in the carbon content (C1s) during enzyme immobilization supported the successful fabrication of monolayer based protease functionalized MNPs (Figure 2c). The grafting of ATRP-initiator, that is required for the SI-ATRP of PGMA, at the surface of MNPs-NH2 was validated by the appearance of signals for bromine at 255 eV, 182 eV and 70 eV for Br3s, Br3p and Br3d respectively. The fabrication of PGMA brushes on the surface of MNPs was verified by the disappearance of Br signals and substantial decrease in the N1s signal intensity as PGMA does not contain any nitrogen that further led to a decrease in N/C ratio from 0.078 in MNPs-Br to 0.02 in MNPs-PGMA (Figure 2d). Immobilization of protease on MNPs-PGMA surface was confirmed by the increase in surface nitrogen content as revealed by the higher content of the N1s signal with a concomitant increase in N/C ratio to 0.082 (Figure 2e).

The magnetization of MNPs under an applied magnetic field is known to decrease with an increase in thickness of surface coating because of the shielding effect. The unmodified MNPs exhibited highest magnetization, which decreased in case of monolayer functionalized MNPs. The polymer brushes functionalized MNPs showed lowest magnetization that can be related to an increased shielding effect inherent with the thick polymer brush layer. The hysteresis loop traces for the magnetization of MNPs under applied magnetic field fully corroborated the MNPs surface functionalization (Figure 2f).

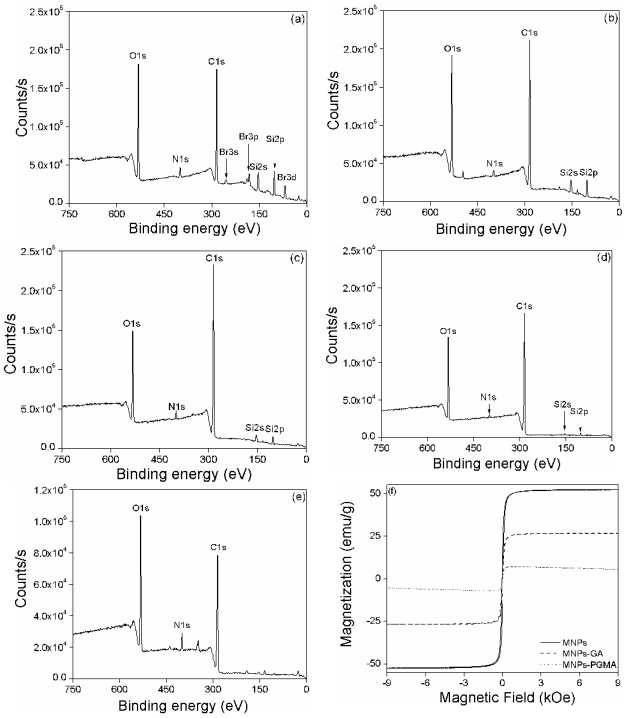

Figure 2. XPS survey scan MNPs-BI (a), MNPs-GA (b), MNPs-GA-Pro (c), MNPs-PGMA (d), MNPs-PGMA-Pro (e) and magnetization curves for MNP, MNP-GA and MNP-PGMA (f).

The SEM and TEM imaging of MNPs revealed ~100 nm size particles with sharp and clean edges (Figure 3a). A thin layer of ~5 nm thickness was observed covering the surface of monolayer functionalized particles (MNPs-GA), whereas the functionalization with PGMA polymer brush led to a thicker layer of ~20 nm uniformly coating the surface of nanoparticles. The higher amount of material deposited at the surface of polymer brushes functionalized nanoparticles is also supported

by the thermogravimetric analysis (TGA, Figure SI-2). In TGA, the pristine MNPs did not show any significant mass change. The GA functionalized MNPs loses about 1.17% of its mass between when heated to 900°C. MNP-Ga-Pro exhibited slightly higher (2.30%) weight loss, which can be attributed to the surface immobilized protease. On the other hand, a much higher amount of organic content was evident from the TGA analysis for polymer brush coated MNPs. MNPs-PGMA showed a significantly higher weight loss (66.72%), while MNPs-PGMA-Pro exhibited total weight loss of 70.3%. From the difference in weight loss, it can be inferred that the enzyme content of MNPs-PGMA-Pro is 3.2 times higher than the enzyme content of MNPs-PGMA.

The MNPs-GA showed a zeta potential of −11.67 ± 1.13 mV. A slightly higher zeta potential (-14.11 ± 1.02) was observed for MNPs-GA-Pro, which could be due to the higher negative charge imparted to the surface by the immobilized enzyme. In case of MNPs-PGMA, the immobilization of protease enzyme on the polymer brush layer enhanced the particle dispersion more drastically and increased the zeta potential from -10.24 ± 0.75 to -17.20 ± 1.05. The enzyme immobilization on MNPs significantly reduced the hydrodynamic size of the MNPs reflecting on increase in surface hydrophilicity and better dispersion in aqueous medium. The increase of zeta potential and decrease of the MNP hydrodynamic size can be related to the enzyme diffusion layer on the MNP surface. Before enzyme immobilization monolayer GA and PGMA brush coated MNPs form large aggregates as observed by SEM images (Fig. 3c and d). However, immobilization of enzyme on the surface possibly decreases the agglomeration of MNPs leading to smaller hydrodynamic diameter and higher zeta potentials.

The stability and activity of enzymes largely depend on temperature therefore the effect of temperature on the activity of free and immobilized protease was studied at different temperatures, ranging from 25°C to 80°C (Figure SI-3). The maximum activity of free (43.14 U/ml) and immobilized enzyme (MNPs-GA-Pro, 45.27 U/ml and MNPs-PGMA-Pro, 47.6 U/ml) was observed at 37°C. Above 40°C the activity of both free and immobilized proteases started to decline.

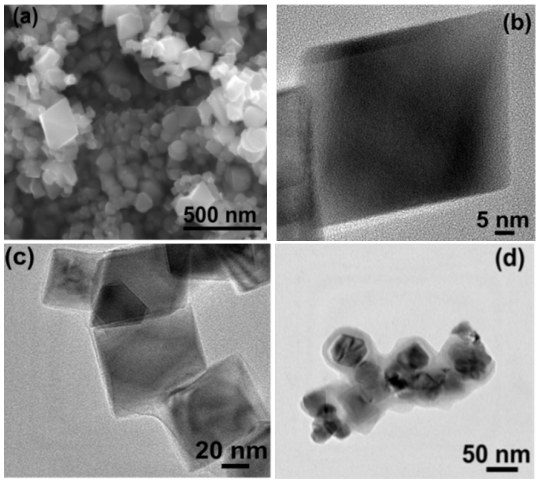

Figure 3. SEM Images MNPs (a), TEM images MNPs-GA (c), MNPs-PGMA (d), and HRTEM MNPs (b).

At 60°C immobilized enzyme (MNPs-GA-Pro and MNPs-PGMA-Pro) retained more than 65% of their activity while the free enzyme showed only 26% of its activity. At 80°C, both MNPs-GA-Pro (23%) and MNPs-PGMA-Pro (35%) exhibited noticeable catalytic activity, whereas the free protease was completely inactive at this temperature. This suggests that polymer brush based platform (MNPs-PGMA-Pro) was more effective in retaining enzymatic activity over the temperature range of 25°C to 80°C.

pH of medium has significant impact on activity of enzymes. The activity of free and immobilized protease (MNPs-GA-Pro and MNPs-PGMA-Pro) was assessed between pH range of 5-12 at 37°C (Figure SI-4). The results showed that activity of free protease was more adversely affected with the change in pH as compare to immobilized enzyme. Maximum activity of free and immobilized proteases was found at pH = 7.0. Above and below pH = 7.0 the activity of enzyme started to decline. At pH 5.0 the activity of free proteases was only 25% while immobilized protease retained 51% (MNPs-GA-Pro) and 63% (MNPs-PGMA-Pro) of activity. As observed in the effect of temperature variation, polymer brush based platform (MNPs-PGMA-Pro) demonstrated better performance over the wide range of pH as compared to the free enzyme.

Bioactivity of free and immobilized proteases was investigated against nematode species *Haemonchus contortus*. Nematocidal activity reveals that about 80%, 84% and 94% of the nematodes were dead after 48 h with free MNPs-GA-Pro and MNPs-PGMA-Pro, respectively. In the case of control (without enzyme) experiment, bare MNPs, and particles with intermediate functionalizations (MNPs-NH2, MNP-GA, MNP-PGMA) did not show any reasonable activity, and >80% nematodes were alive with intact cuticle even after 48 h (Table SI-1).

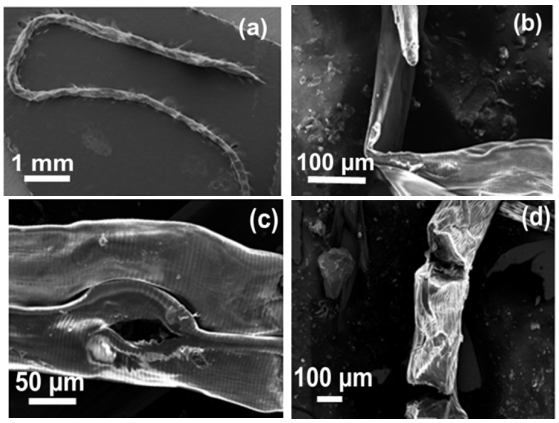

Figure 4. Bioactivity of MNPs-GA-Pro particles on *Haemonchus contortus.* (a) Alive Nematode in Control Sample (b) Nematode were dead after 12 h and cuticle start shrinking (c) On treatment for 24 h holes were appeared on cuticles (d) After 48 h body divided into fragments.

On treatment with MNPs-GA-Pro nematodes were dead after 12 h, their cuticle started shrinking followed by appearance of holes on cuticle within 24 h, and after 48 h their body divided into fragments (Figure 4).

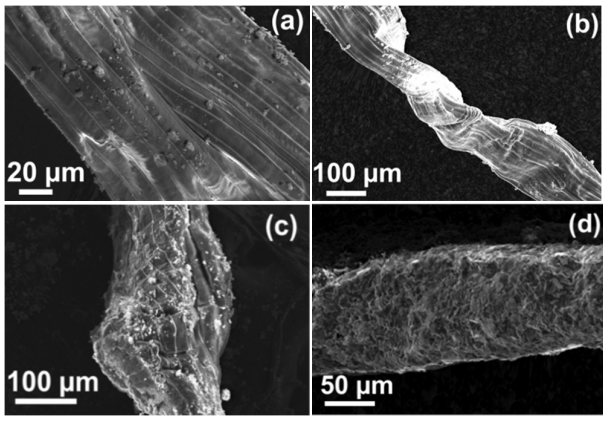

Figure 5. Bioactivity of MNPs-PGMA-Pro particles on *Haemonchus contortus*. (a) Healthy nematodes in control (b) Nematode were dead curve develop after 12 h (c) Cuticle start rupturing after 24 h (d) Cuticle completely ruptured after 36 h

In case of MNPs-PGMA-Pro particles nematode were dead after 12 h and cuticle started to rupture after 24 h, followed by complete destruction of cuticle in 36 h (Figure 5). Therefore polymer brush grafted particles not only manifested enhanced stability but also demonstrated reasonably higher nematocidal activity.

The advantage of immobilizing protease enzyme on MNPs surface is attributed to their reusability via facile magnetic separation. The reusability of immobilized protease was examined up to 7 catalytic cycles (Figure 6). After 3 cycles, the activity of MNPs-GA-Pro started to decline rapidly, while polymer brush functionalized MNPs-PGMA-Pro particles retained reasonable activity even after 7 cycles of magnetic separation. This gradual decrease in activity may be attributed to reversibility of imine bonds that may lead to the dissociation of surface immobilized enzyme over time and/or denaturation of enzyme.[21] The loss of particles during separation process and desorption of any physisorbed enzyme may also contribute towards decrease in the activity.

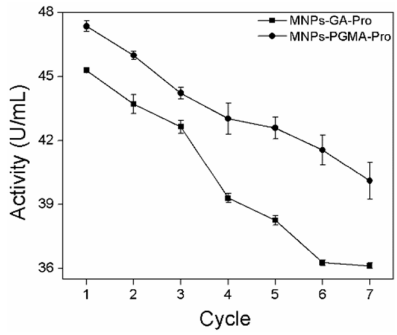

Figure 6. Reusability of immobilized protease for *Haemonchus contortus*.

4. Conclusions

In present work, nematocidal activity of protease immobilized on the surface of MNPs is reported. To compare the effect of surface functionalization methodologies on the enzymatic activity, the surface immobilization of enzyme was carried out via monolayer and polymer brush routes. Compared to free protease, the protease covalently immobilized on the surface of MNPs (MNPs-PGMA-Pro and MNPs-GA-Pro) showed higher nematocidal activity and exhibited greater stability over a wide pH and temperature range. The facile magnetic separation of enzyme functionalized MNPs from their water suspension provided a provision of enzyme reusability. Because of the higher surface functional group density, protease immobilized on the surface of polymer brush functionalized MNPs showed superior enzymatic activity over 7 cycles of magnetic separations when compared to the monolayer based platform. The presented work provides an effective strategy for the future development of magnetically separable and reusable antimicrobial platform that may be potentially employed for water remediation.

Acknowledgements

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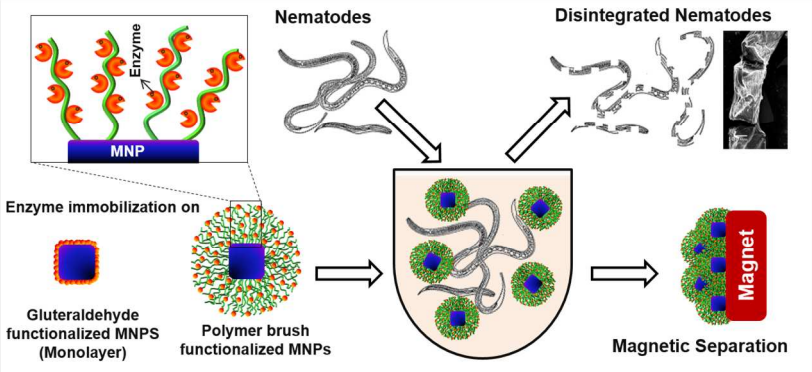

Graphical Abstract