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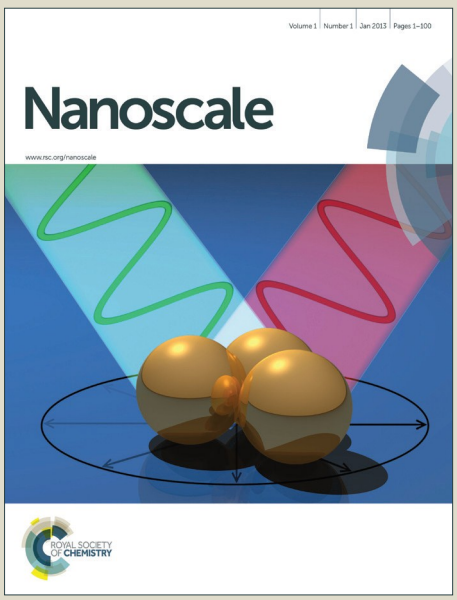

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Synthesis and application of glycoconjugate-functionalized magnetic nanoparticles as potent anti-adhesion agents for reducing enterotoxigenic *Escherichia coli* infections

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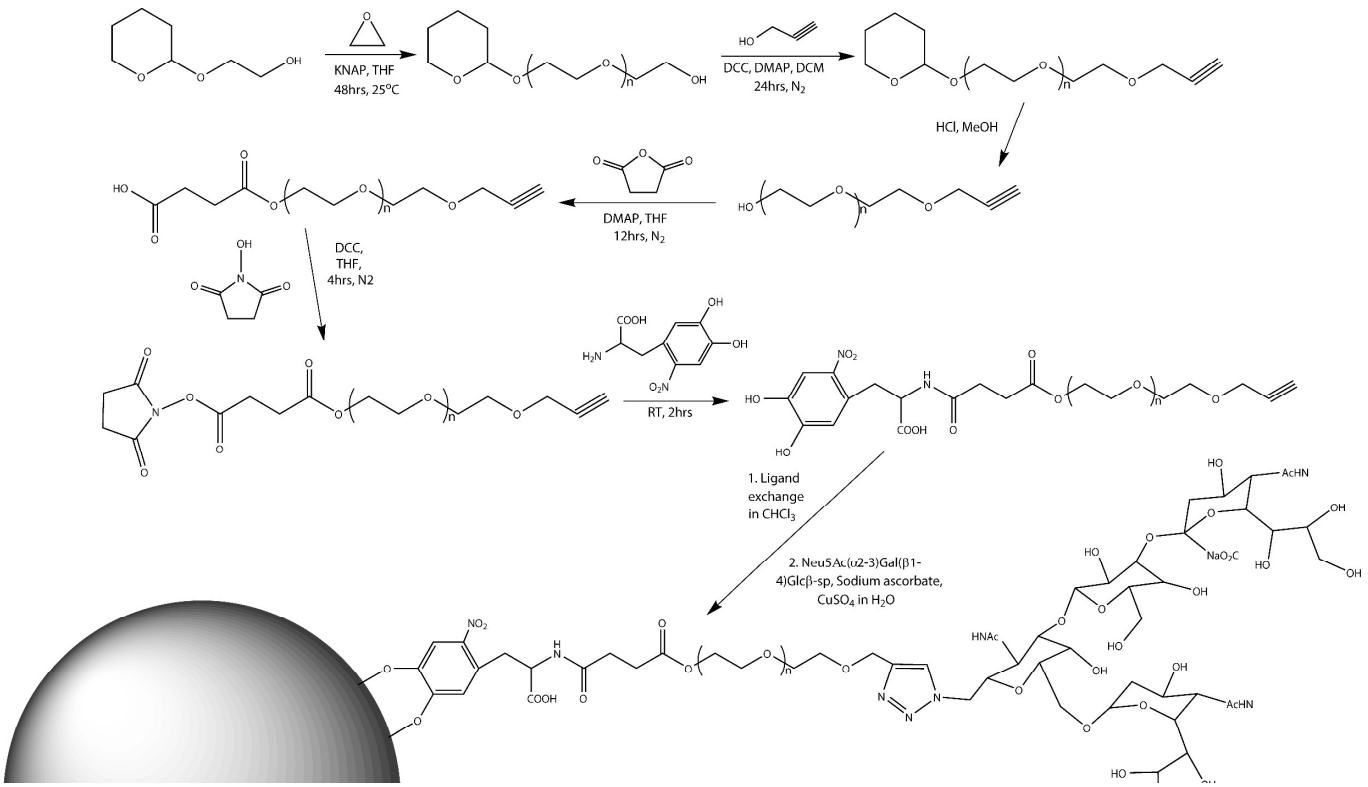
Polyethylene oxide stabilized magnetic nanoparticles (PEO-MNPs) bio-functionalized with glycoconjugate (Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp) (GM3-MNPs) are synthesized using click chemistry. Interaction of GM3-MNPs with Enterotoxigenic *Escherichia coli* (ETEC) strain K99 (*EC* K99) is investigated using different microscopic techniques. Our results suggest that GM3-MNPs can effectively act as non-antibiotic anti-adhesion agents for treating ETEC infections.

There has been a recent uprising in the emergence of new multi-drug resistant bacterial strains in the environment, which has resulted in increased morbidity and mortality throughout the world.[1-3] As a result, alternative therapeutic options that are non-antibiotic based are urgently needed to treat such bacterial infections. There have been considerable ongoing scientific interests in understanding multivalent carbohydrate-lectin interactions for various purposes like receptor mimicking, inhibiting bacterial growth and as novel anti-adhesion agents for treating bacterial infections.[4-8] Functionalizing multivalent carbohydrate molecules onto the surface of different nanomaterials offers numerous advantages, e.g., higher affinity constants (K_a) and enthalpy of binding (ΔH),[9, 10] than their monovalent forms in studying ligand-receptor interactions.[11] Nonetheless, there have been limited reports of using such carbohydrate-functionalized nanomaterials as specific anti-bacterial and anti-adhesion agents. Due to high surface/volume ratio of nanoparticles, it is relatively easy to attach various carbohydrate moieties onto their surface, which has found useful applications in rapid pathogen/toxin detection and its inhibition.[12-14] In several studies, gold nanoparticles,[15] magnetic nanoparticles,[16] carbon nanotubes,[17, 18] polymeric nanoparticles,[19-21] and diamond nanoparticles[22] were bio-functionalized with various carbohydrate sugars and used as mimicking agents of host-cell surface receptors that selectively interacted with the adhesin molecules of various *E. coli* strains and resulted in rapid agglutination[20] and reduction in colony forming units (CFUs) of these *E. coli* strains.[21] The unique magnetic properties and biocompatibility displayed by magnetic nanoparticles have been extensively utilized in wide range of biomedical applications like MRI imaging,[23] pathogen detection,[24] drug-delivery,[25] and magnetic hyperthermia.[26] Keeping MNPs stable in biological environments is important in their biomedical applications. Numerous polymer anchor groups like amines,[27] alcohols,[28] phosphates,[29] and carboxylic acids[30] have been utilized to render colloidal stability to MNPs in highly protein-rich biological environments. Of late, anchor groups based on catechols have been extensively researched to provide stable platform for synthesizing multifunctional MNPs.[31-34] In our previous research, we have demonstrated that gold nanoparticles bio-functionalized with specific sialic-acid sequences can cause rapid aggregation of *EC* K99.[35] Here, we extend our work and further study the intricate details of glycoconjugate receptor binding specificity of GM3-MNPs towards *EC* K99 by employing various microscopic techniques and bioassays and show that these MNPs can effectively act as novel non-toxic anti-adhesion agents in reducing ETEC infections.

Bacterial pathogens utilize two primary mechanisms to adhere onto the host-cell namely carbohydrate-protein recognition and protein-protein interaction.[36] Bacterial adhesin molecules, which take part in carbohydrate-protein interaction, bind bacterial pathogens onto the host-cell tissue through specific glycolipid or glycoprotein receptors.[37] Enterotoxigenic *Escherichia coli* (ETEC) infection is one of the most common cause of traveler's diarrhea in humans and also in neonatal farm animals like calves, pigs and lambs.[38] Recently, numerous studies have reported increases in multi-drug resistance of ETEC associated with antibiotic treated animal feed.[39-42] Majority of the fimbrial adhesins isolated from ETEC that infected farm animals expressed one or more unique adhesins. These adhesins also act as antigens/virulence factors.[42] *EC* K99 is the main causative agent of bloody diarrhea in young calves, lambs and pigs, a condition also known as colibacillosis. This strain bears K99 antigens, which also act as fimbriae facilitating the adherence of *EC* K99 onto the ileal villus epithelium of calf and pigs and help in initiation of infection.[43, 44] The adhesins of *EC* K99 specifically attach to small intestinal mucus and recognize sialic acid derivatives present on glycolipid receptors.[4, 45] Hence, if this attachment is disrupted, then there is high probability of preventing bacterial infection.



Scheme 1. Synthesis of heterobifunctional polyethylene oxide coated magnetic nanoparticles bio-functionalized with sialic-acid derivative (Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp).

The use of bacterial adhesin-specific glycoconjugate functionalized nanoparticles for prevention/treatment of infections offers several advantages in that it can be designed to target only a specific strain or a specific group of pathogens; it does not impose selection pressure on bacteria exposed to it, hence, minimizing the emergence of resistant bacteria; it is more stable and less expensive than antibodies-based targeting systems, etc. Here, we report the synthesis of MNPs coated with heterobifunctional polyethylene oxide (PEO-MNPs) having nitroDOPA as a stable anchoring agent and bio-functionalized with sialic-acid glycoconjugate (Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp) (GM3-MNPs) via 'click chemistry'(scheme 1). These GM3-MNPs can effectively act as multivalent ligands, which specifically interact with adhesins present on the *EC* K99. A heterobifunctional polyethylene oxide (PEO), with a molecular weight of 6300 g/mol, with a protected alcohol on one end and an alcohol on the other was synthesized by the anionic polymerization of ethylene oxide (EO) using tetrahydropyranol as an initiator. The alcohol end group was then modified with an alkyne via a substitution reaction using propargyl bromide (1:4 respectively). Once modified, the protected alcohol group was deprotected with an acid and subsequently purified. Finally, using *N*-hydroxysuccinimide (NHS) and *N,N'*-dicyclohexylcarbodiimide (DCC) coupling, the heterobifunctional PEO was modified with nitrated 3,4 dihydroxy-L-phenylanaline (nitroDOPA) to yield a macromolecule with functionality that can be utilized for 'click chemistry' and provide enhanced binding to an iron oxide surface, as described in previous work by Stone et al.,[46] where synthetic details the polymer formation and relevant NMR data can be found (Supplementary information). NitroDOPA was selected as the binding group for our system because of its enhanced binding to metal oxides.[47]

The aforementioned macromolecule was then used to modify MNPs, synthesized using a modified version of a procedure by Sun et al.,[48] via ligand-exchange by slowly adding magnetic nanoparticles dispersed in hexanes to a solution of the macromolecule in chloroform while sonicating (SI). After 12 hours, allowing for significant ligand exchange, the polymer-particle complex was then purified by extraction into DI water and then dialyzed against DI water in 12-14,000 g mol-1 MWCO dialysis membranes for three days to remove any impurities. The use of nitroDOPA containing PEO polymer has recently been shown to be the most effective anchoring chemistry in the ligand exchange of oleic acid coated particles.[49] The particles with an alkyne surface were then modified using 'click chemistry' with glycoconjugate (Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp) and azide, 1:4 respectively using a copper catalyst for 24 hours in the absence of light. The particles were then dialyzed for 3 days to remove any unbound glycoconjugate, catalyst, and any byproducts of the reaction. The resulted particles with attached glycoconjugate (GM3-MNPs) were characterized by dynamic light scattering (DLS) (Table S1), zeta-potential (Table S1) and FT-IR spectroscopy (S2 and S3). The absence of azide peak at 2113 cm^{-1} in Fig. S3(D) represents the successful conjugation of glycoconjugate moiety onto the polymer-coated magnetic nanoparticles through

cycloaddition. The nanoparticle concentration in PEO-MNPs and GM3-MNPs was determined by means of inductively coupled plasma mass spectroscopy (ICP-MS) (SI).

Both *EC* K99 (ATCC 13762) and non-virulent strain *E. coli* O157:H7 6980-2 (*EC* 6980-2; control strain) were transformed with plasmids pGREEN and pGFPuv respectively by electroporation.[50] The green-fluorescent protein (GFP) expressing *E. coli* strains were grown in tryptic soy broth/tryptic soy agar (TSB/TSA) supplemented with ampicillin (100 μg/ml). Freshly grown *E. coli* cultures were used for aggregation assays. After growth, the bacterial cells were centrifuged thrice and re-suspended in 1X sterile phosphate buffered saline (PBS). Approximately, 5 x 10^7 CFU of bacterial cells were prepared based on optical density (OD_{600}) readings. PEO-MNPs (40 μg/ml) and GM3-MNPs (40 μg/ml) were mixed with both *EC* K99 and *EC* 6980-2 and this mixture was incubated at room temperature for 30 minutes with gentle shaking. Based on previous method,[21] fluorescence microscopy assays was performed at the end of incubation time to visualize nanoparticles-mediated bacterial aggregation.

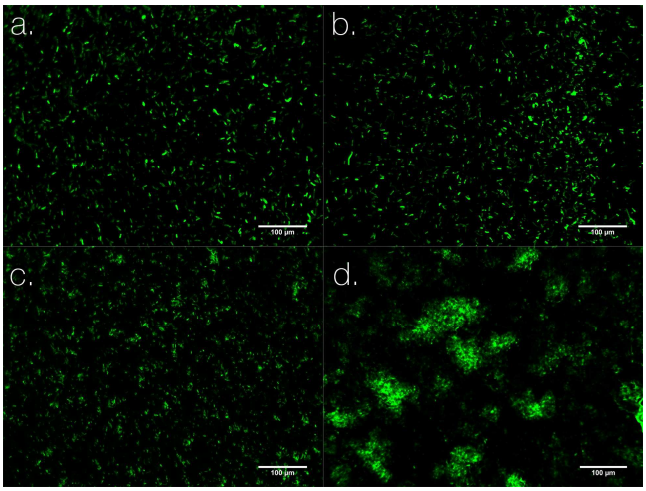


Figure 1. Fluorescent microscopy images of *EC* 6980-2 and *EC* K99 in presence of PEO-MNPs and GM3-MNPs. (a) and (b) *EC* 6980-2, in the presence of PEO-MNPs and GM3-MNPs respectively; (c) and (d) *EC* K99, in the presence of PEO-MNPs and GM3-MNPs respectively. Magnification – 400X and scale bar – 100 μm.

Clearly, large aggregates of *EC* K99 were observed in the presence of GM3-MNPs (Fig. 1d). No visible aggregation was found when *EC* K99 was mixed with PEO-MNPs (Fig. 1c). Moreover, *EC* 6980-2 did not form aggregates when mixed with PEO-MNPs and GM3-MNPs (Fig. 1a and 1b). This shows that both *E. coli* strains clearly have different sugar binding specificities and that *EC* K99 exhibits binding specificity only to GM3-MNPs. Furthermore, transmission electron microscopy (TEM) analysis of the mixture containing GM3-MNPs and *EC* K99 was done to observe intricate details of bacterial aggregation. The samples for TEM analysis were prepared according to a modified multi-step protocol (SI).[21, 51] Large clusters of bacterial cells were observed due to strong interactions between GM3-MNPs and *EC* K99 (Fig. 2a and 2b). The GM3-MNPs were found covering the entire surface of *EC* K99. Because of significant binding of multiple GM3-MNPs onto individual bacterial cells and other GM3-MNPs acting as linking agents to interact with other bacterial cells, we observed significantly large agglutination of *EC* K99. Entero-pathogenic *E. coli* strains have different sugar binding affinities depending on the type of adhesins present on their outer cell-surface.[52] It is known that *EC* K99 has S type of fimbrial proteins that specifically recognizes and binds only to Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp sialic-acid sequences.[4] The pilus of *EC* K99 is primarily made up of FanC, major fimbrial sub-unit gene product, which is responsible for recognizing and attaching the bacterium to the sialic-acid ganglioside receptors present on the host-cell.[53] On the other hand, *EC* 6980-2 have adhesin molecules that consist of galactose-binding proteins on its outer surface which can attach to galactose receptors[17] present on the host-cell and hence *EC* 6980-2 was not able to form bacterial aggregates when mixed with GM3-MNPs. Based on these sugar-binding specificities, the above mentioned 2 different *E. coli* strains were selected for this study.

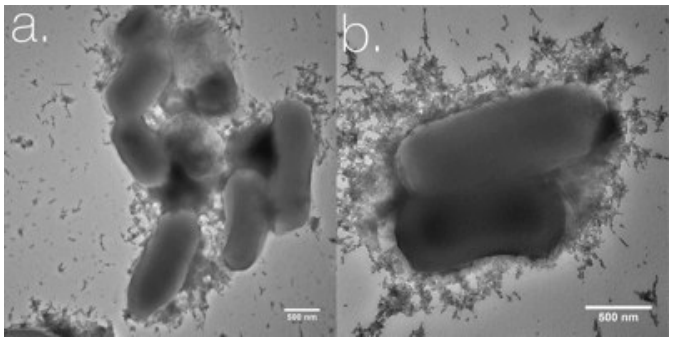


Figure 2. TEM images of GM3-MNPs induced bacterial aggregation of *EC* K99. Magnification of (a) and (b) are 30000X and 60000X, respectively. Scale bar is 500 nm.

In order to determine the extent of GM3-MNPs induced bacterial aggregation, a CFU reduction assay was carried out as previously described.[21] Briefly, both the *E. coli* strains were standardized to a concentration of 5 x 10^7 CFU in 1X PBS. These bacterial suspensions were then mixed with different amounts of PEO-MNPs and GM3-MNPs (40 μg/ml and 100 μg/ml) and the mixture was allowed to incubate at room temperature for 30 minutes with gentle shaking. Serial dilutions of these mixtures were made and 50 μl of sample from each dilution tube was transferred onto a sterile, empty petri plate in triplicates. Then, 20 ml of molten TSA (maintained at 45°C) supplemented with ampicillin (100 μg/ml) was carefully poured into the petri plates. The plates were gently rotated to ensure proper mixing of sample and TSA. Finally, after the TSA in the plates solidified, the plates were incubated at 37°C overnight. Next day, the colonies on the plates were counted and CFU reduction was compared to control plates.

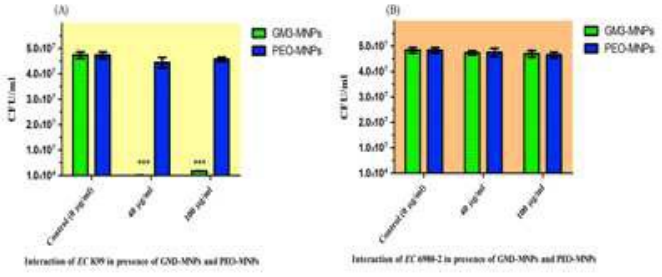


Figure 3. (**a**) CFU/ml assay of *EC* K99 in the presence of different concentrations of GM3-MNPs and PEO-MNPs and (**b**) CFU/ml assay of *EC* 6980-2 in the presence of different concentrations of GM3-MNPs and PEO-MNPs. Data expressed as Mean ± SD (n = 3); Statistical Analysis – Two-Way Analysis of Variance (ANOVA) (*** - P<0.0001).

As shown in Fig. 3a, approximately 2-log reduction in CFU of *EC* K99 was observed in the presence of GM3-MNPs (40 μg/ml). This reduction was due to GM3-MNPs induced bacterial aggregation. Also, 1-log reduction of *EC* K99 was observed at 100 μg/ml concentration of GM3-MNPs. Interestingly, there was no reduction in CFU of EC 6980-2 in the presence of either GM3-MNPs or PEO-MNPs (Fig. 3b). These results correlate with those obtained in fluorescence microscopy assay showing that bacterial cells of *EC* K99 aggregating in clusters of several 100s of bacterial cells in the presence of GM3-MNPs. Surprisingly, *EC* K99 in the presence of GM3-MNPs (100 μg/ml) resulted only in 1-log reduction in CFU since the nanoparticle-bacteria ratio was different. Luo and co-workers[21] obtained similar results when they incubated *E. coli* ORN178 in the presence of different concentrations of mannose-functionalized polymeric nanoparticles. Thus, it is imperative to attain appropriate nanoparticle-bacteria ratio for getting maximum reduction in CFU mediated by nanoparticles-induced bacterial aggregation.

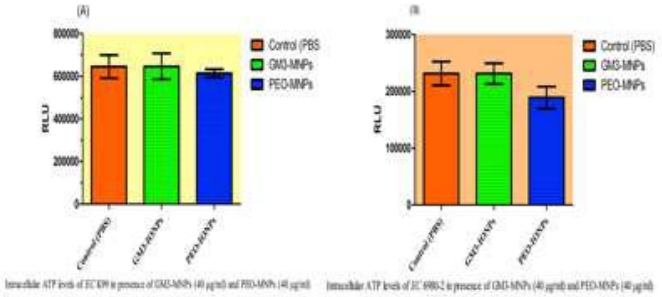


Figure 4. Intracellular ATP Assay **(a)** Relative Luminescent Units (RLU) of *EC* K99 when incubated with GM3-MNPs and PEO-MNPs (**b)** RLU of *EC* 6980-2 when incubated with GM3-MNPs and PEO-MNPs. Data expressed as Mean ± SD (n = 3); Statistical Analysis – One-Way Analysis of Variance (ANOVA); (P>0.05).

To further confirm that the reduction in CFU of *EC* K99 was achieved only due to GM3-MNPs induced bacterial aggregation and not because of inherent toxicity of these nanoparticles, an ATP determination assay[54] was performed using BacTiter-Glo microbial cell viability kit (Promega, Madison, WI) following manufacturer's protocol. Intracellular ATP levels of both the *E. coli* strains were recorded by measuring their relative luminescence in the presence/absence of GM3-MNPs (40 μg/ml) and PEO-MNPs (40 μg/ml). Fig. 4a and 4b shows that there is no significant difference in intracellular ATP levels of both *E. coli* strains in the presence of nanoparticles. Also, another important feature to determine the toxicity of nanoparticles is to look for any cell-membrane damage and morphological changes in the bacterial cell-membrane structure.[51] Results of TEM imaging of *EC* K99 in the presence of GM3-MNPs showed no visible bacterial cell-membrane damage suggesting the non-toxic nature of our nanoparticles. These results suggest that the reduction in CFU of *EC* K99 was achieved only because of GM3-MNPs induced bacterial aggregation and not due to nanoparticle toxicity. To further validate the non-toxic nature of our nanoparticles system, we performed a preliminary cytotoxicity assay on human colon (normal) cell-line CCD-18Co and determined its cell-viability rate after exposing to GM3-MNPs for 24 hours (Suppl. Info). The potential cytotoxicity of GM3-MNPs was measured using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) kit purchased from Promega, USA. As seen in Fig. 5, there was no significant reduction in cell-viability rate of CCD-18Co cells in the presence of varying concentrations of GM3-MNPs. These results suggest that our novel glycoconjugate-functionalized nanoparticle system is highly biocompatible.

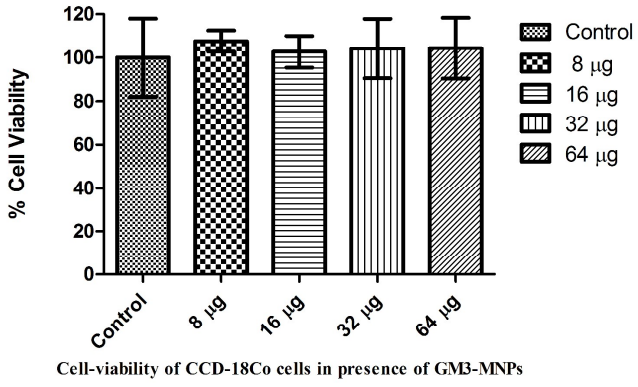


Figure 5. Cytotoxicity assay to determine cell-viability of CCD-18Co cells in presence of different concentrations of GM3-MNPs. Data expressed as Mean ± SD (n = 3); Statistical Analysis – One-Way Analysis of Variance (ANOVA); (P>0.05).

Conclusions

In conclusion, we successfully synthesized heterobifunctional polymer coated magnetic nanoparticles that have nitroDOPA as a stable anchoring agent and were bio-functionalized with sialic-acid glycoconjugate (Neu5Ac(α2-3)Gal(β1-4)Glcβ-sp) (GM3-MNPs) using click chemistry. The GM3-MNPs were characterized by employing different techniques and their adhesin specificity was

determined using aggregation assays. Our GM3-MNPs specifically interacted only with ETEC strain *EC* K99 as confirmed through fluorescence microscopy and transmission electron microscopy. Also, a 2-log reduction in CFU of *EC* K99 was achieved due to GM3-MNPs induced bacterial aggregation. Moreover, intracellular ATP assays demonstrated that the 2-log reduction in CFU of *EC* K99 was not due to inherent toxicity of the nanoparticles. Further, the preliminary cytotoxicity assay results on human cell-line proved the highly biocompatible nature of our nanoparticles system. Thus, our proof-of-concept nanoparticle system can effectively serve as novel non-antibiotic multivalent carriers, which could find applications in detection and capturing of pathogenic multi-drug resistant bacterial strains from active physiological body fluids. Our systems can especially reduce/treat gastro-intestinal tract infections caused by ETEC pathogens in farm animals and humans since specific bacterial-nanoparticle aggregates can be effectively flushed out from the body system because of high peristaltic flows without disturbing the normal gut microflora that is usually destroyed when antibiotics are used. This system can also be employed as potent anti-adhesion agents that can block/inhibit specific cellular responses by competitively preventing the attachment of bacterial pathogens onto specific eukaryotic cell-surface receptors and thereby reducing the infection load. Furthermore, this nanoparticle system can also be utilized for targeted magnetic hyperthermia treatment of bacterial infections, especially those that are resistant to multiple antibiotics. In future work, nanoparticles with multi-anchored functional groups will be utilized to improve stability of nanoparticles in biological fluids and to enhance their bindings to specific pathogens. Their therapeutic values, i.e., selective killing of pathogens via hyperthermia mediated by glycoconjugate-functionalized magnetic nanoparticles, will be evaluated both *in vitro* in cell-line and *in vivo* in small animal systems. In addition, potential toxicities associated with the use of these nanoparticles will be characterized using various biological assays, e.g., cytotoxicity, genotoxicity, immunogenicity assays, etc.

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Notes and references

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Electronic Supplementary Information (ESI) available: Materials and methods used in the synthesis and characterization of the polymer and particles described in this manuscript See DOI: 10.1039/c000000x/

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