Scientific paper

Toxicity of Magnetic Chitosan Micro and Nanoparticles as Carriers for Biologically Active Substances

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Abstract

Nanoparticles of inorganic magnetic core surrounded by layers of functional coatings are potential representatives of nanostructures for immobilization of bio-substances. Magnetic nanoparticles (MNPs) are often bound in aggregates due to a strong magnetic dipole, which has a lot of advantages, such as large surface area for binding biologically active substances. Chitosan is a polysaccharide polymer that is non-toxic, hydrophilic, biocompatible and has hydroxy and amino groups in its structure. Because of these chemical and biological properties it is a desirable bio-product for immobilization of enzymes and for binding of other biologically active substances. Magnetic micro and nanoparticles were synthesized with chitosan by three different methods; microemulsion process, suspension cross-linking technique and covalent binding of chitosan. Toxic effect of the prepared magnetic particles was determined as well and was examined on five different bacterial cultures; *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterococcus faecalis* and *Klebsiella pneumoniae*. At concentrations of 10–30 mg of magnetic particles per 0.5 McFarland Standard solution of *E. coli* and per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis in K. pneumonia*, no inhibition on the chosen bacterial cultures was detected.

Keywords: Magnetic nanoparticles, chitosan, binding of biologically active substances, toxicity tests

1. Introduction

Applicability of MNPs and bio substances has become a great interest of research in different scientific disciplines, including biotechnology, biomedicine, biochemical engineering, and magnetic fluids etc.,[1–4] due to several advantages in bio substance binding. With the recent growing interest in nanotechnology, particle size has shrunk by 2–3 orders of magnitude and the applications for nanoparticles have expanded (Figure 1). First, nanoparticles were used in detection systems and in other research procedures. Inorganic nanoparticles were also extensively used in various bioapplications. For example, gold nanoparticles have been used as detection labels for immunohistochemical (IHC) staining and lateral flow diagnostic testing.[5] Magnetic nanoparticles are typically crystals of inorganic elements for which the largest characteristic dimension is approx. 1–100 nm. Their inorganic magnetic core is usually surrounded by layers of functional coatings.[3,6,7,8] Due to their strong magnetic dipole, they have a large surface area on which different active substances can bind, such as chitosan,[8–12] DNA complexes[13] etc. They also have a high coercivity and high magnetic susceptibility and are often bound together in aggregates. Magnetic nanoparticles are also used as carriers for delivering different targeting drugs into desired tissue.[1,14] They are often used in medicine for diagnostic, since they are a good contrast device for magnetic resonance (MR) imaging. MNPs are able to concentrate in a specific tissue or anatomical location. Results of clinical studies[15] may help us to determine the key substances for immobilization on MNPs with the aim to gain better control of 4 healing processes: migration and proliferation of fibroblasts, deposi-

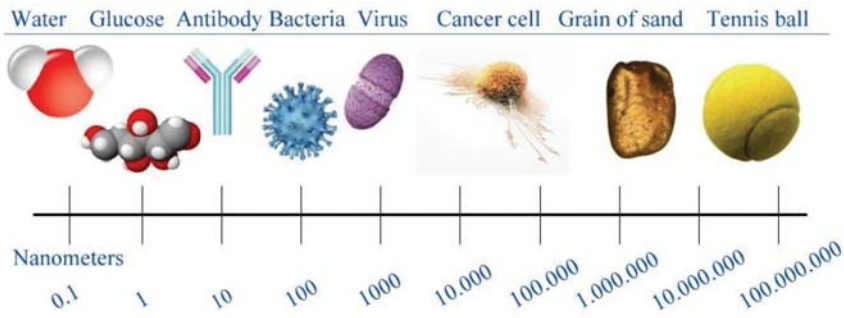

Figure 1: Nanoparticle scale

tion of extracellular matrix, angiogenesis and transformation – scarring.

Chitosan is a polysaccharide polymer which is produced by deacetylation of chitin. It is non-toxic, hydrophilic, biocompatible, biologically degradable and antibacterial and has a hydroxy and amino group in its structure. Because of its biological and chemical properties, chitosan is a desirable biomaterial used for immobilization of enzymes and binding of other biologically active substances. It has the ability to provide optimal micro environment and is able to contain high biological activity and stability. For preparation of magnetic chitosan nanoparticles, three different methods were used. Microemulsion process with dissolving of chitosan in 2% acetic acid, in the presence of emulsifier and gluteraldehyde; suspension cross-linking technique with dissolving of chitosan in 5% acetic acid and in the presence of emulsifier and gluteraldehyde; and with a method of covalent binding of chitosan dissolved in milliQ water without emulsifier and gluteraldehyde. Since magnetic nanoparticles are potential representatives for bio substances immobilization and activation of bioactive substances, their toxic effect was determined on five different bacterial cultures. Three Gram negative bacteria were chosen, *E. coli* as most commonly tested bacteria, *K. pneumoniae* because of its cell capsule and *P. aeruginosa* as a non-fermenting bacteria; and two Gram positive bacteria, *S. aureus and E. faecalis. E. coli* is a bacteria from genus *Escherichia*, a Gram-negative, rod shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. *S. aureus* is from genus *Staphylococcus* which is a facultative anaerobic Gram-positive coccal bacterium. It is frequently found as a part of normal skin flora on the skin and nasal passages. *P. aeruginosa* is from genus *Pseudomonas*, which is a Gram-negative, aerobic, rod-shaped bacterium with unipolar motility. *E. faecalis* from genus *Streptococcus*, is a Gram-positive, commensal bacterium inhibiting the gastrointestinal tracts of mammals. *K. pneumoniae* is a member of *Klebsiella* genus from *Enterobacteriaceae*. It is a Gram-negative, facultatve anaerobic, lactose fermenting, encapsulated, rod shaped bacterium, found in the normal flora of the mouth, skin and intestines. Present paper reviews the applicability of MNPs with the goal of providing preliminary data for medical purposes, therefore only toxicity tests on bacterial cultures were performed. Nanoparticles are mostly mentioned to be used for drug delivery, while microparticles can be applicable in chemical reactions with certain types of reactors. Many articles describe all these applications,[1–31] but the present paper investigates the applicability of all MNPs coated with chitosan.

2. Materials and Methods

2. 1. Materials

Chitosan (MMW, 75–85%), gluteraldehyde (GA) and Span–80 were obtained from Sigma-Aldrich (Germany), parafine oil from Pharmachem (Slovenia), acetic acid (AC), ferric (Fe^{2+}) and ferrous (Fe^{3+}) ions from Merck (Germany), ATCC standards for bacteria from Clinical and Laboratory Standards (USA), TSB (tryptic soy broth), bovine blood agar and all five bacteria, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Enterococcus faecalis* from Institute of Public Health Maribor (Slovenia).

2. 2. Preparation of Magnetic γ-Fe_2O_3 (maghemite)

Magnetic nanoparticles of maghemite were prepared with the process of co-precipitation of ferrous (Fe^{2+}) and ferric (Fe^{3+}) ions. Droplets of ammonium were slowly added to the previously prepared ferric and ferrous ions solution. The solution was mixed and pH value was adjusted from 3 to 11. Magnetic nanoparticles were collected on a magnet and dried in a drier at 50 °C.[16] Later magnetic γ-Fe_2O_3 nanoparticles were coated with citric acid to stabilize magnetic γ-Fe_2O_3 nanoparticles. The suspension was heated under stirring at 75 °C and later centrifuged at 5000 rpm.[16]

2. 3. Preparation of Magnetic Chitosan Nanoparticles With Microemulsion Process

Chitosan was dissolved in acetic acid (2%) in aqueous solution and magnetic γ-Fe_2O_3 nanoparticles were dispersed into the solution under ultrasonic stirring for

30 min. Paraffin oil and emulsifier Span-80 were added into the mixed system under mechanical stirring at room temperature. The dispersion was then stirred in a water bath at 40 °C for 60 min and gluteraldehyde was added. pH value of the solution was adjusted to 9–10 by using 1mM NaOH. Afterwards the temperature was raised and kept at 70 °C for another 60 min. Nanoparticles were then collected on a magnet and dried at 50 °C under atmospheric conditions.[10]

2. 4. Preparation of Magnetic Chitosan Nanoparticles with Suspension Cross-linking Technique

Magnetic γ-Fe_2O_3 nanoparticles were dispersed in a solution with paraffin oil and emulsifier Span-80. Then acetic acid (5%) in aqueous solution was added and the solution was mixed by ultrasonic stirring for 30 min, while chitosan was added to the solution. After addition of gluteraldehyde the solution was mechanically stirred for another 4 hours. Nanoparticles were collected on a magnet and dried at 50 °C under atmospheric conditions.[11]

2. 5. Preparation of Magnetic Chitosan Nanoparticles with Covalent Binding of Chitosan

Chitosan was dissolved in milli Q water, magnetic γ-Fe_2O_3 nanoparticles were added to the solution and pH was adjusted to 3.7. The solution was stirred on ultrasonic bath for 60 min at 60 °C and further mechanically stirred for 12 hours. Nanoparticles were collected on a magnet and dried at 50 °C under atmospheric conditions.[12]

2. 6. Toxicity Tests of Magnetic Nanoparticles Prepared by Three Previously Described Methods on Selected Bacterial Cultures

Toxicity or the degree to which a substance can damage an organism was determined on five different bacterial cultures for maghemite magnetic nanoparticles and chitosan coated magnetic nanoparticls prepared by all three different described methods. For toxicity determination American Type Culture Collection (ATCC) standards for all five bacteria were used. For each toxicity test, 10–30 mg of maghemite magnetic or chitosan coated magnetic nanoparticles were used.

2. 6. 1. Toxicity Tests on Bacteria E. Coli

In the case, where toxic effect of magnetic nanoparticles on bacteria *E. coli* was examined, 10–30 mg of chitosan magnetic nanoparticles were applied directly on Mueller-Hinton agar with suspension of culture *E. coli*. 0.5 McFarland Standard solution of bacteria *E. coli* was used. The bacterium growth was observed after 24 hours and 96 hours at concentratios of 10–30 mg of chitosan magnetic nanoparticles per 0.5 McFarland Standard solution of culture *E.coli*.

2. 6. 2. Toxicity Tests on Bacteria S. Aureus, P. Aeruginosa, E. Faecalis and K. Pneumoniae

In case, where toxic effect of magnetic nanoparticles on bacteria *S. aureus, P. aeruginosa, E. faecalis and K. pneumoniae* was examined, 10–30 mg of chitosan magnetic nanoparticles together with bacterial cultures of 400 CFU were applied in tryptic soy broth (TSB) liquid media and incubated at 35 °C. Liquid media of approx. 0.5 mL was grafted on petri dish with bovine blood agar after 1, 5, and 10 days. Bacterial growth was then observed after 24 hours at concentrations of 10–30 mg of chitosan magnetic nanoparticles per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* cultures.

2. 7. SEM Analysis

Scanning electron microscopy (SEM) is used to analyse the surface of materials where a focused beam of high-energy electrons generates a variety of signals at the surface of solid samples. The signals that derive from electron-sample interactions reveal information about the sample including external morphology (texture), chemical composition, and crystalline structure and orientation of materials making up the sample. It produces representations of three-dimensional samples from a diverse range of materials and measures and evaluates surface pitting, failure analysis, characterization of dust, deposits, contaminants, particles, filter residues, and other applications.[17–19]

The morphology of the magnetic nanoparticles and magnetic nanoparticles coated with chitosan were investigated using SEM (FE SEM Sirion, 400 NC, FEI) equipped with Energy Dispersive System (EDS). The samples for SEM analysis were covered with a thin layer of conducting material, such as gold, to prevent the accumulation of electrostatic charge at the surface and analyzed to determine particle size and form.

2. 8. TEM Analysis

Transmission electron microscopy (TEM) is with its atomic-resolution real-space imaging of nanoparticles a unique technique for structure characterization.[20] TEM (HARTEM, JEOL 2110F) was used to determine the range of particle size and morphology of nanoparticles. TEM microscope was equipped with LaB_6 electron beam source, thermal emission and CCD camera. The material for TEM analysis was dispersed in deionized water and placed on copper grids covered with a perforated carbon film to dry at room temperature.

3. Results and Discussion

3. 1. SEM Analysis

Morphology of uncoated and with chitosan coated maghemite magnetic nanoparticels was investigated using SEM to observe the surface of the material. Figure 2 represents SEM images of uncoated maghemite nanoparticles (a), chitosan coated magnetic nanoparticles prepared with microemulsion process (b), chitosan coated magnetic nanoparticles prepared with suspension cross-linking technique (c) and chitosan coated magnetic nanoaprticles prepared with covalent binding of chitosan (d). Figure 2 (a) shows maghemite nanoparticels with their average size around 22.78 nm, (b) and (c) shows the round-spheric shape of magnetic microparticles and their size, which is 40–350 μm and 10–50 μm, respectively. The size of chitosan coated magnetic nanoparticles, prepared with covalent binding of chitosan, is between 50 and 100 nm (Figure 2d).

The SEM micrograph of chitosan coated magnetic nanoparticles prepared with microemulsion process (Figure 2b) indicates that so prepared magnetic nanoparticles were composed of abound unequal microparticles with outer diameter of 40–350 μm. With this technique the largest particle size of the chitosan coated maghemite nanoparticle was obtained, indicating that magnetic maghemite nanoparticles have been encapsulated by chitosan. The SEM micrograph of chitosan coated magnetic microparticles prepared with suspension cross-linking technique (Figure 2c) shows that there is a little aggregative phenomenon in the particles. Their size is between 10–50 μm, which is smaller than in previous case, but still not in nm size.

The SEM micrographs of uncoated magnetic γ-Fe_2O_3 nanoparticles and chitosan coated magnetic micro and nanoparticles, prepared by three described methods, also indicate the spherical shape of all magnetic particles. When preparing chitosan coated magnetic nanoparticles with covalent binding of chitosan (d), a big difference can be noticed in particle size compared to other two methods. This is due to preparation of nanoparticles without cross-linking agent GA in the process of covalent binding of chitosan. GA was used as a cross-linking agent in both microemulsion process (b) and suspension cross-linking technique (c), which contributes to a bigger nanoparticle size.

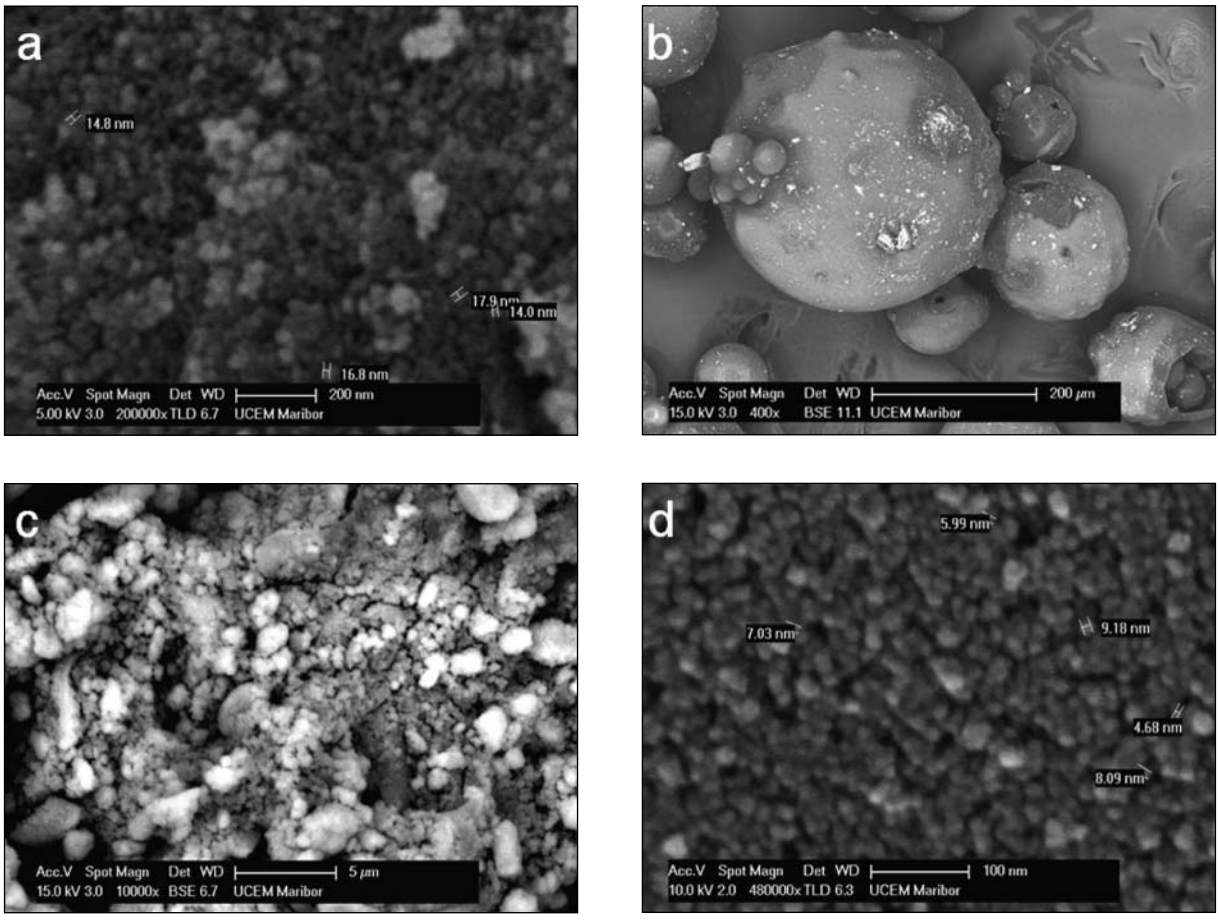

Figure 2: SEM of different magnetic particles; uncoated maghemite nanoparticles (a), chitosan coated magnetic nanoparticles prepared with microemulsion process (b), chitosan coated magnetic nanoparticles prepared with suspension cross-linking technique (c) and chitosan coated magnetic nanoaprticles prepared with covalent binding of chitosan (d).

3. 2. TEM Analysis

TEM measurements of uncoated maghemite MNPs were performed and described before and show a continuous layer of silica on the surface of the maghemite core, where the estimated thickness is 3 nm.[21] In Figure 3 the TEM micrograph shows the amorphous layer of chitosan that surrounds the γ-Fe_2O_3 nanoparticles. TEM data of maghemite nanoparticles coated with chitosan by microemulsion process show polydisperse, spherical particles where the layer of chitosan around the maghemite nanoparticles is thicker than the layer around maghemite nanoparticles prepared by the method of covalent binding of chitosan. This can be compared to our previous work and reveals the same observations.[22]

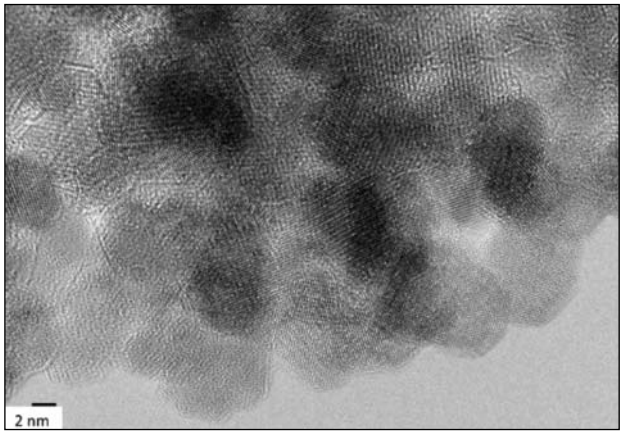

Figure 3: TEM image of amorphous layer of chitosan that surrounds the γ-Fe_2O_3 nanoparticles

3. 3. Toxicity Tests

Inhibition of uncoated magnetic maghemite nanoparticles and with chitosan coated magnetic micro and nanoparticles on bacterial cultures of *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa, Enterococcus faecalis* and *Klebsiella pneumoniae* was studied. Therefore, 10–30 mg of magnetic particles were applied on bacterial culture of 0.5 McFarland Standard solution of *E. coli* and the bacterial growth was observed after 24 and 96 hours of incubation.

As can be seen in Figure 4 none of the investigated magnetic micro and nanoparticles inhibited the growth of bacteria *E. coli* culture at concentrations of 10–30 mg of chitosan magnetic nanoparoticles per 0.5 McFarland Standard solution of *E.coli* culture, since even after 96 hours the bacterial growth proceeded.

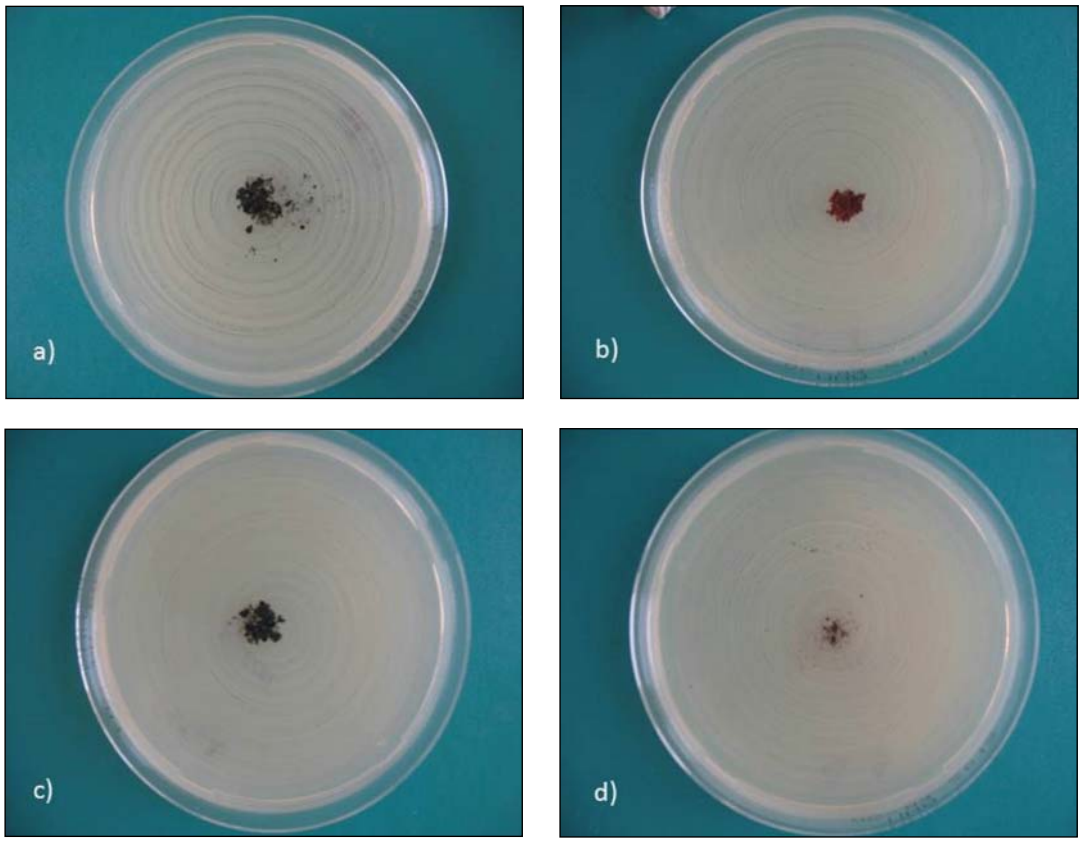

Figure 4: Uncoated maghemite MNPs (a), chitosan coated magnetic micro particles, prepared with microemulsion process (b), chitosan coated magnetic micro particles, prepared with suspension cross-linking technique (c) and chitosan coated MNPs, prepared with covalent binding of chitsan (d) on bacterial culture of *E. coli* after 96 hours.

At the second series of toxicology tests, 10–30 mg of maghemite magnetic particles were applied together with 400 CFU of bacterial cultures of *S. aureus, P. aeruginosa, E. faecalis* in *K. pneumoniae* in liquid TSB media and incubated for 1, 5 and 10 days. After incubation at 35 °C the solution was grafted on bovine blood agar and the bacterial growth was observed after 24 hours. Inhibition of magnetic micro and nanoparticles on bacterial cultures of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* did not occure at the concentrations of 10–30 mg of magnetic micro and nanoparticles per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis and K. pneumoniae* cultures, since bacterial growth proceeded even after 10 days of incubation. The results of the above mentioned toxicology tests indicate that uncoated maghemite MNPs and with chitosan coated magnetic micro and nanoparticles, prepared by three different methods, microemulsion process, suspension cross-linking technique and covalent binding of chitosan, have no toxic effect on the selected bacterial clutures of *E. coli, S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* at given concentrations of 10–30 mg of uncoated maghemite MNPs and with chitosan coated magnetic micro and nanoparticles per 0.5 McFarland Standard solution of *E.coli* culture and per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* cultures.

Figure 5 presents maghemite nanoparticles and different chitosan magnetic micro and nanoparticles prepared by three different methods, microsemulsion process, suspension cross-linking technique and process of covalent binding of chitosan on bacterial cultures of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae.* It is evident that none of the prepared magnetic micro and nanoparticles inhibited any of the selected bacterial cultures at given concentrations of 10–30 mg of each maghemite or chitosan coated magnetic nanoparticles per 0.5 McFarland Standard solution of *E.coli* and per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* cultures.

There are some studies on toxicity of chitosan nanoparticles without magnetic properties[23,24] which report biological interactions between human liver cells and chitosan nanoparticles, which have been widely recognised as biocompatible. Toxicity data on chitosan nanoparticles in correlation to the in situ surface characteristics of those nanoparticles are described, as well.[25] But there are limited studies which determine toxicity of chitosan magnetic nanoparticles on other organisms. As few report,[25] chitosan magnetic nanoparticles were tested for toxic effects on a mouse embryonic fibroblast cell line (3T3) using the MTT method. The toxic concentration of magnetic nanoparticles was reported to be within the testing range which

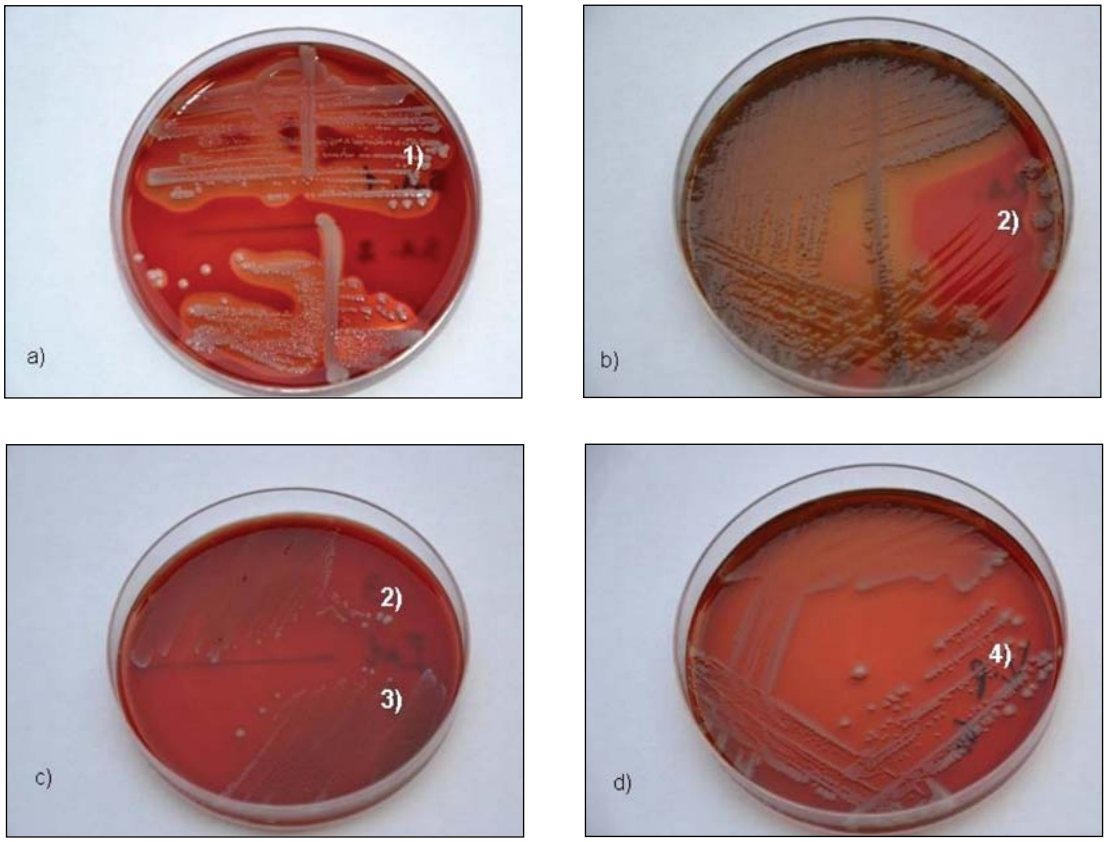

Figure 5: Maghemite MNPs (1), magnetic chitosan micro and nanoparticles prepared with: microemulsion process (2), suspension cross-linking technique (3) and covalent binding of chitosan (4) on bacterial cultures of *S. aureus* (a), *P. aeruginosa* (b), *E. faecalis* (c) and *K. pneumoniae* (d) in bovine blood agar.

indicated that chitosan magnetic nanoparticles elicited no cytotoxicity. Toxicity of magnetic nanoparticles on biological entities is also highly dependant on structural properties, dosage and the intended usage.[26–28] There have been several toxicity mechanisms of magnetic iron oxide nanoparticles described, suggesting high chemical reactivity with reactive oxygen species (ROS) when cells are exposed to nanoparticels at high doses. Used techniques to assess toxicity are *in vitro* assays for proliferation, microscopic analysis of intracellular localization, *in vitro* hemolysis and gene express analysis. Those *in vitro* systems are useful for identification of specific characteristics of iron oxide nanoparticles as indicators of toxicity. *In vivo* systems are more dynamic and complicated, since the adsorption of the iron oxide nanoparticles with the body can occur through interactions with cells and proteins and later distribute into different organs. Also blood compatibility is important, where lack of blood compatibility can result in coagulation.[29]

Specific receptors on the cell surface provide useful targets for nanoparticles, so therapeutic compounds or image enhancement agents can be delivered very precisely. This allows us to increase the effect, and in particular reduce the side effects of the active substances. Magnetic targetnig using iron oxide core enables us local retention for greater effect. This might be an option to deliver specific molecules to an anatomical location. Animal studies are encouraging.[30] Still in its youth nanomedicine or clinical application of nanotechnology is still far from common use. Biological effects of these materials on humans is not well documented. Degradation and accumulation of heavy metals needs additional research. Animal studies might point towards our next steps. Advances in this field are considered one of the most promising areas of future medicine.[31]

4. Conclusions

Chitosan coated magnetic micro and nanoparticles were prepared by three different methods: microemulsion process, suspension cross-linking technique and covalent binding of chitosan. They were placed on bovine blood agars to provide a sufficient evidence of their toxic effect on five different bacterial cultures. All three types of magnetic micro and nanoparticles were observed during an extended period of time. Chitosan coated magnetic micro and nanoparticles did not inhibit the bacterial growth of the selected bacteria. During the research, no inhibition was detected. Results have shown, that none of the prepared magnetic nanoparticles inhibited any of selected bacterial cultures in giving concentrations, which ware 10–30 mg of maghemite and chitosan coated magnetic nanoparticles per 0.5 McFarland Standard solution of *E.coli* culture and 10–30 mg of maghemite and chitosan coated magnetic nanoparticles per 400 CFU of *S. aureus, P. aeruginosa, E. faecalis* and *K. pneumoniae* cultures. By that we can conclude, that magnetic micro and nanoparticles prepared by the three different methods described before, have no toxic effect on bacterial cultures at selected concentrations, which makes them a suitable, biocompatible and non-toxic carrier for biologically active substances, applicable in further research. As micro particles are not applicable in systems for drug delivery, they can widely be used in different types of reactors, involving different chemical reactions. For that purposes toxicity of magnetic micro and nanoparticles was studied. Our next step is to estimate the impact of chitosan on the biological activity of selected substances. The main objective in our future research would be binding of selected bioactive substances and study of their activity thereafter. Key elements in biological processes must be in the right conformational forms to participate. Whether or not this is possible in association with nanoparticles is to be further explored.

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Povzetek

Nanodelci iz magnetnega anorganskega jedra, oblečeni z večslojno funkcionalno prevleko, predstavljajo pomemben razred nanostrukturiranih delcev za vezavo biosubstanc. Zaradi močnega magnetnega dipola se magnetni nanodelci pogosto združujejo v skupke, kar pa ima številne prednosti, kot na primer velika površina, na katero lahko pritrdimo različne biološke komponente. Hitozan je polisaharidni polimer, ki je nestrupen, hidrofilen, biokompatibilen in vključuje prisotnost hidroksilne in amino skupine v svoji strukturi. Zaradi naštetih kemijskih in bioloških lastnosti spada hitozan med zaželjene biomateriale za imobilizacijo encimov in vezavo drugih biološko aktivnih substanc. Magnetne nanodelce, prevlečene s hitozanom, smo pripravili po treh različnih postopkih; s postopkom mikroemulzije, s postopkom suspenzijske zamreževalne tehnike ter s postopkom kovalentne vezaven hitozana. Toksikološke vplive pripravljenih magnetnih delcev smo preverili na petih različnih bakterijskih kulturah; *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecalis* in *Klebsiella pneumoniae*. Pri koncentracijah 10–30 mg magnetnih delcev na 0.5 McFarland standardne raztopine bakterijske kulture *E.coli* in na 400 CFU bakterijskih kultur *S. aureus, P. aeruginosa, E. faecalis* in *K. pneumonia* do inhibicije rasti mikroorganizmov ni prišlo.