RESEARCH ARTICLE

In vitro biocompatibility of low and medium molecular weight chitosan–coated Fe_3O_4 nanoparticles

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ABSTRACT

Objective(s): The chitosan- Fe_3O_4 core- shell nanoparticles were synthesized. The nanoparticles should be coated properly in the shape of core-shell, so that they remain hidden from the body's immune system after coating. Effects of different molecular weight in coating were investigated.

Methods: Nanoparticles coated with low and medium molecular weight chitosan were synthesized in one step. In this way, first, the nanoparticles were prepared by co-precipitation method. Then, the surface of the nanoparticles was modified using oleic acid. Finally, the nanoparticles were coated with low or medium molecular weight chitosan. The properties of particles were investigated by TEM, XRD, VSM and FT-IR devices as well as the Debye Scherrer method. In biocompatibility study, the nanoparticles were transferred to a medium containing fibroblast cells which were extracted from the mouse embryo and cultivated in an incubator. Then, dead and live fibroblast cells were counted.

Results: The growth of fibroblast cells that were adjacent to the nanoparticles were different. The percentage of live cells in the container containing uncoated particles in the sixth day was 20%. Also, the percentage of live cells in a container containing particles covered with low and medium molecular weight chitosan were 90 and 98% in the sixth day respectively.

Conclusions: The molecular weight of chitosan can have a significant effect on the toxicity of nanoparticles in biological environments throughout time, so it shows that the medium cells containing particles coated with medium molecular weight chitosan had better growth than low molecular weight chitosan coated particles.

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INTRODUCTION

Today, the uses of nanoparticles have led to diverse applications in the aircraft, medical, automotive, sewage treatment industries, due to its desirable properties as well as its small size. Generally, nanoparticles are used in both medical diagnosis and treatment [1]. For example, the production of morphine detection sensors [2], drug delivery to a specific tissue in the body[3], imaging, or treatment of cancer tumors [4] and many others can be named. Various strategies, such as surgery, radiation therapy, chemotherapy, etc. are used to treat cancer tumors. One of these uses is the detection and treatment of cancer tumors

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using super paramagnetic nanoparticles. By using these nanoparticles, cancer cells are labeled, thus depending on the target, the diagnostic process or treatment is done. One of the methods that have always been considered as a non-invasive treatment is hyperthermia [5, 6]. Though, nanoparticles are used to produce heat in the cancerous tumor and surrounding areas, thereby preventing the delivery of food and oxygen to cancer cells, and finally, the death of cancer cells will happen under the influence of temperature. One of these nanoparticles which used in hyperthermia is Fe_3O_4.

Fe3O4 is usually produced by chemical methods, and the resulting particles are generally spherical and a size of about 8 to 20nm is reported [7-9] .Various methods are used to prepare these nanoparticles, each of which depends on many parameters. For example, the microemulsion [10,11] and co-precipitation [12] and so on. Since uncoated nanoparticles are generally unstable, they need to be stable by a suitable coating. This will improve their chemical stability, allowing more mass flow particles into the bloodstream, preventing the oxidation of nanoparticles, increasing biocompatibility and preventing the particle from accumulation and blockage of the veins. For this purpose, the pure nanoparticles should be coated properly in the shape of core-shell, so that they remain hidden from the body's immune system after coating. This coating can be made of biocompatible polymers such as chitosan [4] or their combination with ceramics such as hydroxyapatite [13] and others. The coating can be done in one-step [12] or two-steps [4,9]. In two steps, pure nanoparticles produce, then dry in the oven and add to the solution containing the coating. However, in one step, production and coating of nanoparticles are carried-out continuously. The advantages of one step coating are high-speed coating and preventing agglomeration of the particles.

In hyperthermia, Fe_3O_4 nanoparticles are coated with chitosan which is a natural polymer and a cationic polysaccharide and is prepared by the process of deacetylation of chitin. Thus it has different molecular weights [14]. This linear polysaccharide composed of D-glucosamine amines (D units) and N-acetylglucosamine (A units) which is distributed randomly in the state of β (1-4). It is a biodegradable polymer, and oral intake is safe for humans [15]. Nanoparticle coatings with chitosan have been studied in several papers, in which nanoparticle powder was first produced, then the nanoparticles were functionalized and surface-modified, and then the powder was added to the solution containing chitosan. It should be noted that the chitosan used in these studies has molecular weight of 1.4 × 105 to 4.9 × 105 [12, 16, 17]. The thickness of the coating on nanoparticles varies from 1.7 nanometers to 7.38 nm in different molecular weight chitosan [9, 12]. In some cases, the size of the coated particle between 10 and 30 nanometers has also been reported [18,19].

The fibroblast cells are the main connective tissue cells for the production of collagen and mucopolysaccharides . Fibroblasts are a type of cell that synthesizes cellular and collagen matrices. For various research, these cells are extracted from various organisms. For example, these cells can be extracted from the embryo of the mouse [20], whale [21] and sometimes from goat [22]. The researchers used these cells for in vitro studies, and it has always been tried before using nanoparticles inside the body, first, these cells are placed in a laboratory medium, after obtaining the proper results of the desired nanoparticles, they are used in the body of the animal and then in the human body [23].

Choosing the appropriate molecular weight from chitosan biopolymer is an important strategy for the preparation of Fe_3O_4 - chitosan core - shell nanoparticles suitable for hyperthermia applications. In the present study, nanoparticles coated with low and medium molecular weight chitosan were synthesized in one step. In this way, first, the nanoparticles were prepared by co-precipitation method. After modification of the surface of the nanoparticles with oleic acid, the nanoparticles were coated with low and medium molecular weight chitosan. Then, fibroblast cells of the mouse were extracted and cultivated, and the coated nanoparticles containing low and medium molecular weight chitosan were placed in the culture medium and in the incubator in the vicinity of them. After the desired times, the percentage of live cells were evaluated and the results were announced. Then, the properties of the nanoparticles were examined and indicated that the thickness of the shell formed on the nanoparticle and also the molecular weight of the chitosan has a direct effect on the amount of saturation magnetization of the super paramagnetic nanoparticles. Also, the

molecular weight of chitosan can affect the growth of fibroblast cells over time. So that the particle growth curve with low molecular weight chitosan coating has a smaller magnification factor than particles with medium molecular weight chitosan coatings. However, coating particles with medium molecular weight chitosan polymer can reduce the pure particle toxicity. It can be used this Fe_3O_4-chitosan nanoparticles in cancer treatment and the other application in medicine.

The new work in this research is the use of different molecular weights of chitosan and study of the effect of molecular weight on the growth of fibroblast cells, saturation magnetization and shell thickness formed on nanoparticles.

MATERIALS AND METHODS

Materials

Ferric chloride hexahydrate ($FeCl_3.6H_2O$), ferrous chloride tetrahydrate ($FeCl_2.4H_2O$), acetic acid, acridine orange, EMDM culture medium, oleic acid, glutaraldehyde and poly (vinyl pyrrolidone) (PVP) had merck brand and chitosan with low and medium molecular weight was purchased from sigma company.

Coating of Fe_3O_4 nanoparticles with chitosan in one step

In the first stage, in a 250 ml balloon, 60 ml of deionized distilled water was poured out. The second stage, the amount of 0.72g NaOH was added. At this stage, 0.3 g PVP was added to the contents of the balloon to prevent the nanoparticle from being cracked. Then, the solution is placed on a heater and a magnetic stirrer to reach 90°C. In this period stirrer speed was 1400 rpm. The third stage, a solution of 0.5 molar $FeCl_3$ and 0.5 molar solution of $FeCl_2$ was prepared. The ratio of metal salts was 1 to 1.36. The fourth stage, a 0.5 molar solution of $FeCl_3$ was added to the contents of the balloon. After 5 minutes, the fifth stage was performed, that is, a 0.5 molar solution of $FeCl_2$ was added to the balloon content. At the same time as the last stage was added, a black colored deposit was created at the bottom of the balloon very quickly. So, it took 20 minutes to complete the reactions. In the last stage, 3 ml of oleic acid was added to the synthesized particles of balloons content. Particles modified with oleic acid was kepton a magnetic stirrer for an hour with 1400 rpm at 70°C. The resulting precipitate was collected with a magnet, and the contents of the balloons were washed with deionized water and ethanol for ten times. It should be noted that all of the above reactions were carried out in the vicinity of inert gas N2. Finally, newly synthesized particles which are black liquid sinter and have high viscous, are collected and transferred to a test tube.

To prepare a solution of chitosan with different molecular weights used in this study, 100 ml of deionized water was poured into the balloon. Next, 2 ml of acetic acid was added to it. In the following, 0.5 g of chitosan (once with medium molecular weight and once with low molecular weight) was added to the above solution. Finally, the solution was placed at 40°C on an ultrasonic agitator for 20 minutes.

To complete the process, two 100 ml beakers were placed on an ultrasonic agitator, and in one of them, a chitosan solution containing low molecular weight and in the other a medium-molecular-weight chitosan solution was poured. The ultrasonic agitator temperature was placed at 40°C at this time and it took 20 minutes for the temperature of water inside the stirrer and the content of chitosan inside the balloon were homogeneous and reached 40°C. 20 g of a viscous black liquid solution (prepared in the previous step) was added to each of the above beakers and waited for 15 minutes. 2 ml of glutaraldehyde 20% wt was added to the contents of each beakers. At this time, the ultrasonic agitator temperature is 40 °C and the duration of the connection time is considered about 3 hours. After adding glutaraldehyde, the contents of beakers became a diluted gel solution in which the Fe_3O_4 nanoparticles were uniformly dispersed within the solution. Subsequently, the particles were collected using a magnet at the bottom of the container and two times with deionized distilled water and ethanol were washed. The resulting precipitate is then transferred to a container with a 20 cm span and dried in a furnace under nitrogen gas at 40°C for 72 hours.

Biocompatibility of Fe_3O_4-chitosan nanoparticles

Extraction of fibroblast cells

Fibroblast cells were extracted from a rat embryo that had been two days since its birth as follows [24]. Firstly, the embryo was anesthetized with ether. It was transferred to the syringe and after several times aspiration, the contents of the syringe transferred to the culture medium. Continuously, the culture medium was transferred to the incubator

with standard conditions (37°C, 95% humidity and 5%CO_2).The specimen was placed in an incubator for 48 hours, then the medium was changed and re-inserted into the incubator and for seven days, one day in between, all the medium existing inside the plates were replaced. The newly extracted cells were cultured in a culture medium and their growth was controlled by microscopy. Ultimately, cells with a bright and oval nucleus tend to have a spindle-shaped with 1 to 2 nuclei with branching cytoplasmic that are matching with the morphology of fibroblastic cells as shown in Fig. 5. During this time, the growth of the cells was normal.

Add nanoparticles to the medium containing fibroblast cells

One gram of each nanoparticle; coated (low and medium molecular weight) and non-coated; was poured separately into three test tubes. 5 ml of culture medium was added to each of these test tubes. Subsequently, with using pipet from each of the solutions in the test tube, one and a half ml was added to each of the cells of the culture container containing fibroblast cells.

The container of the medium and the fibroblast cells contained sixteen cells which identified the first column showed the control sample (to compare the effect of the presence of nanoparticles) without nanoparticle, the second column to check on the first day of adding nanoparticles and the third column for the study of the third day, after adding nanoparticles. The forth column was used to examine the sixth day after adding nanoparticles [28].

Characterization of pure Fe_3O_4 and Fe_3O_4-chitosan nanoparticles

The morphology of pure Fe_3O_4 and Fe_3O_4 -chitosan nanoparticles were investigated by transmission electron microscopy (TEM) (Zeiss Germany). X-ray diffraction (XRD) analysis (Equinox French model 3000 with Cu Kα radiation, λ=1.540598 Å) was used for determination of nanomaterial structures. Fourier transform infrared spectrophotometer (FTIR) (Thermo Nicolet -Model Nexus 670 of (USA)) was applied to study of chemical structure changes. Magnetic properties of nanoparticles were investigated by Vibrating Sample Magnetometer (VSM), model CBHV-55, Riken (Japan) and in the biocompatibility section alive and dead cells were evaluated by invert microscope (Leica Germany).

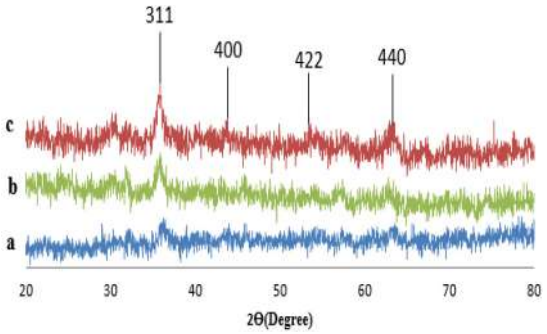

Fig. 1. a)Fe_3O_4-medium molecular weight chitosan, b) Fe_3O_4-low molecular weight chitosan, c)pure Fe_3O_4

RESULTS AND DISCUSSION

XRD characterization

In x-ray diffraction on particles coated with medium molecular chitosan, peaks at angles of about 35.83°,43.27°, 57.26° and 62.98° appeared (Fig. 1a). On the other hand, in a nanoparticle with chitosan coating of low molecular weight, peaks appeared at 36.04°,43.74°, 54.01° and 42.63° angles(Fig.1b). In X-ray diffraction, done on pure particle Fe_3O_4 particles, peaks appeared at 36°, 44°, 54° and 64° angles. According to the reference pattern number 75-0449, it can be seen that the main peaks from synthesized nanoparticles have an angular alignment with the graphs, which confirms the presence of magnetite crystalline phase (Fe_3O_4) in Fig.1c.The particle size is determined by using the Debye Scherer's law according to Table 1. Due to these peaks and also comparing the reference pattern by number 0449-75-01, it can be concluded

Table 1. The size of one-step Fe_3O_4-chitosan particles synthesized according to Debye Scherer's law

	Low molecular weight	Medium molecular weight	Uncoated particles
Particle size	22.38	17.87	13.18

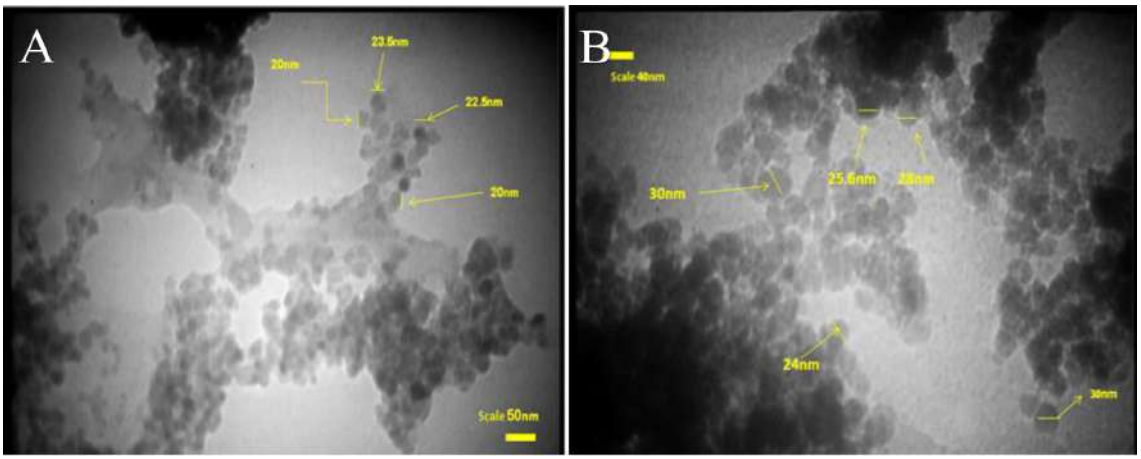

Fig. 2. TEM images A) nanoparticle with low molecular weight chitosan
B) nanoparticle with medium molecular weight chitosan

that surface correction with oleic acid and coating with chitosan cannot change the structure of Fe_3O_4 crystalline structure. These results are in agreement with data that have been reported by other researchers. [18,25]

TEM characterization

The TEM image represents a nanoparticle coated with low molecular weight chitosan as shows in Fig. 2A. The morphology of the particles is spherical and the average particle size is about 21.5nm, and almost all particles have chitosan coatings. In Fig 2B, nanoparticles coated with medium molecular weight chitosan are presented. The morphology of the particles is spherical and the average particle size is about 27.5nm. Considering that in Table 1, the net particle size calculated by Debye Scherer's law is equal to 13.18nm, the average thickness of the coating on nanoparticles with chitosan with medium molecular weight is 7.16nm and chitosan with low molecular weight is 4.16nm.

FTIR characterization

The FTIR spectrum shows Fe_3O_4 nanoparticles prepared with low and medium molecular weight chitosan coatings in single step on curves a and b in Fig. 3. 1711 cm^{-1} and 1713 cm^{-1} bands, respectively, are related to carbonyl and N-H groups and 1440.87 cm^{-1} and 1441.68 cm^{-1} bands are related to the C-O stretch bonding of the first type alcoholic group of chitosan. A sharp band appeared in areas of 1711.67 $cm^{-1}$and 1713.93 cm^{-1}indicating that glutaraldehyde reacted with chitosan. Also, a peak related to Fe-O was observed in588.68 cm^{-1} and 588.70 cm^{-1} areas. As a result, chitosan covers the Fe_3O_4 nanoparticles that show chitosan can coat the Fe_3O_4 nanoparticles by electrostatic reaction and chemical reaction through a glutaraldehyde crosslinker.

In Fig. 3, curve c represents the FT-IR spectrum of Fe_3O_4 nanoparticles corrected with oleic acid. The band of about 1566 cm^{-1} and 3384 cm^{-1} represents the water absorption in the sample. The band in the 584 cm^{-1} region represents Fe_3O_4, which indicates that the correction of the surface has no effect on the nanoparticle. The regions of 2852cm^{-1} and 2922cm^{-1} are oleic acid bands that are related to the symmetric and asymmetric stretch of C-H band of oleic acid, respectively. The 1711 cm^{-1}band is for the oleate group. Region 1093 cm^{-1} refers to the C-O

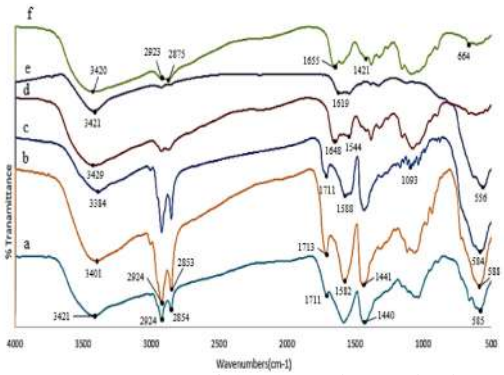

Fig. 3. FTIR spectrum of a) Fe_3O_4-medium molecular weight chitosan, b) Fe_3O_4-low molecular weight chitosan, c) Fe_3O_4-oleic acid, d) medium molecular weight chitosan, e) pure Fe_3O_4, f) low molecular weight chitosan

Table 2. Values of magnetized saturation

Nanoparticle type	measured magnetized saturation emu/g
Pure Fe_3O_4	56.5
Fe_3O_4-low molecular weight chitosan	20.6
Fe_3O_4-Medium molecular weight chitosan	11.7

Table 3. The percentage of live fibroblasts cells at different times

Groups	Percentage of live cells		
	24 hours	72 hours	144 hours
- Control group	85	95	98
- Pure nanoparticles synthesized by co-precipitation method	87	62	20
-Nanoparticle with medium molecular weight chitosan	85	95	98
-Nanoparticle with low molecular weight chitosan	85	88	90

band of oleate. These data indicate that oleic acid is coated on the surface of the Fe_3O_4 nanoparticles. In Fig. 3, curves f and d, the FTIR spectra of pure chitosan with low and medium molecular weights are presented. In this case, the bands of 1544.83 cm^{-1} and 1421.38 cm^{-1} are related to the C-O stretch bonding of the chitosan type I alcoholic group. The 1655.11 cm^{-1} and 1648.94 cm^{-1} correspond to the N-H bending bond. Fig. 3, curve e, shows the FTIR spectrum of the Fe_3O_4 nanoparticles synthesized with coprecipitation method. The band of 556.67 cm^{-1} corresponds to the Fe-O bond, which indicates the formation of Fe_3O_4, and bands of 3421.23 cm^{-1} and 1619.46 cm^{-1} are related to water absorption. In Fig. 3a, b, d and f the broad band around 3400 cm^{-1} is assigned to the stretching mode of the O–H and N–H bonds in the chitosan. These results are in agreement with data that have been reported by other researchers [2,16,18,26].

Vibrating Sample Magnetometer (VSM) characterization

Magnetic saturation in pure nanoparticles synthesized by coprecipitation method, Fe_3O_4 nanoparticles/oleic acid/One-step low molecular weight chitosan and Fe_3O_4 nanoparticles/oleic acid /One-step medium molecular weight chitosan are presented in Table 2 and Fig. 4.

As can be seen, pure nanoparticles have a higher saturation magnetization (56.5 emu/g) than particles coated with two types of chitosan. This difference indicates that the particles after the coating are completely surrounded by chitosan, which has led to a significant drop in the saturation magnetization in the coated nanoparticles. On the other hand, particles coated with low molecular weight chitosan had a saturation magnetization of 20.6 emu/g and particles coated with medium

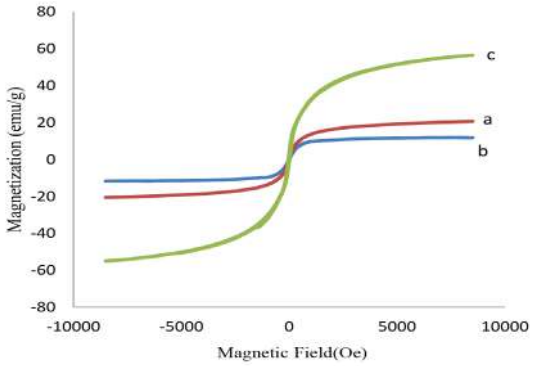

Fig. 4. a) Fe_3O_4-low molecular weight chitosan, b) Fe_3O_4-medium molecular weight chitosan, c) pure Fe_3O_4

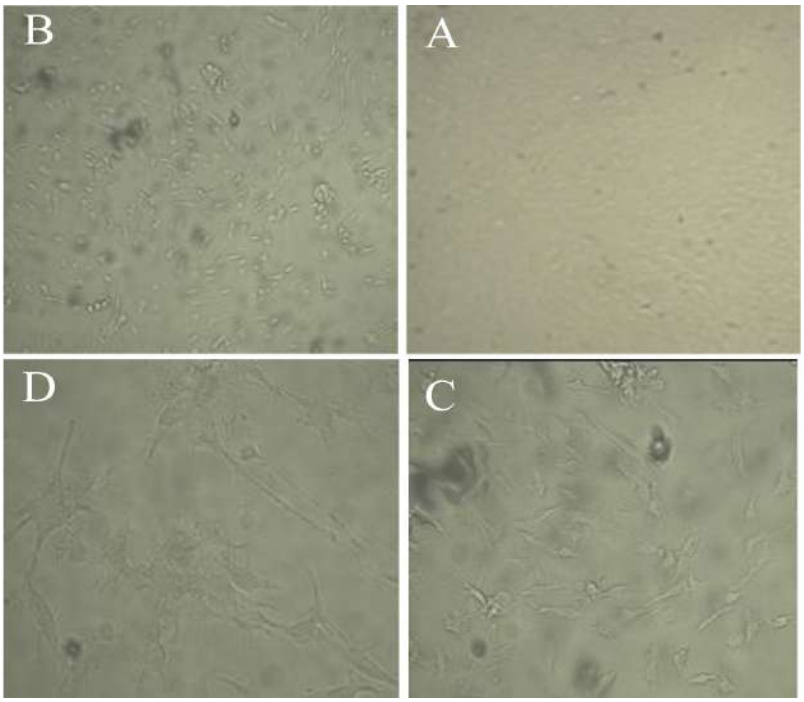

Fig. 5. Invert microscope images of the cultivated fibroblast cells in magnification A)10X, B)20X, C)40X, D)100X

molecular weight chitosan had 11.7 emu/g. The difference in the saturation magnetization may be due to two items, first, the molecular weight, second, the average thickness of coating on the nanoparticles in particles with a medium molecular weight of about 7.16nm and in particles with a low molecular weight is 4.16nm. The effect of both of these items leads to a decrease in the saturation magnetization in a sample with medium molecular weight. These results are in agreement with data that have been reported by other researchers [4,9,17,27].

The medium containing fibroblast cells and nanoparticles

After adding the nanoparticles to the culture medium after 24, 72 and 144 hours respectively, the percentage of live cells was counted with the addition of acridine orange to the medium and transferring the particles to the neobar lam (Table 3).

The growth of fibroblast cells that were adjacent to the nanoparticles was different, so that the percentage of live cells in the container containing uncoated particles on the 1st day was 87%, which reached 62% and 20% on days 3th and 6th, respectively. Also, the percentage of live cells in a container containing particles covered with low molecular chitosan was 85% on the 1st day, reaching 88% and 90% on days 3th and 6th, respectively. On the other hand, this percentage in a container containing particles coated with the medium molecular chitosan was 85% on the first day, reaching 95% and 98% in the third and sixth days, respectively (Fig.5,6).

Cell cultures

Considering the cell population and time elapsed, the most suitable function for mapping the cell population behavior is natural logarithmic functions based on naprin (Fig.7). The logarithmic functions in the initial x have a high growth rate, and after the required time, this growth rate is dramatically constant, so that a horizontal asymptotic can be considered. The magnification factor of these functions is presented as α. This coefficient expresses the elongation and magnitude of the function. As can be seen, in the curve b, the magnification factor of the cell growth function for cells adjacent to the low molecular weight nanoparticles is 5.653, which is smaller in comparison with the magnitude coefficient, of the curve equation of particles with chitosan with the medium molecular weight of 7.257. On the

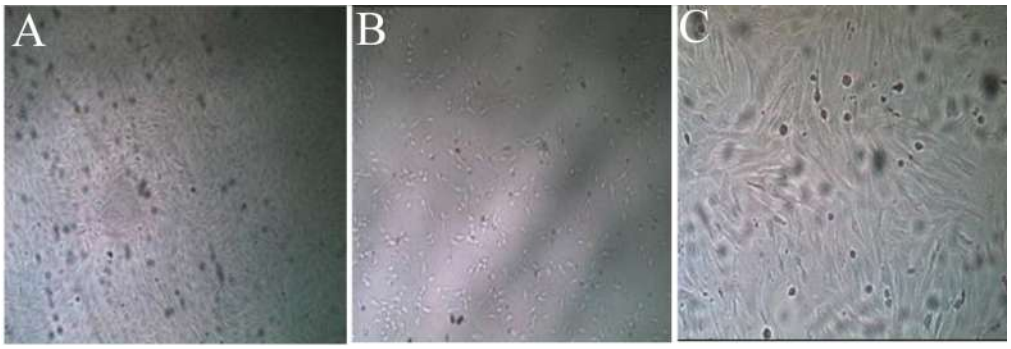

Fig.6 .The culture medium containing nanoparticles. A)10X, B) 20X, C)40X

other hand, the magnification coefficient c, which corresponds to the control group curve, is about 7.423. According to the above magnitudes, it was observed that the magnification coefficients of the curves a and c are close to each other, on the other hand, they did not differ much in the intercept. As expected, the cell population function should show behavior by logarithmic functions. (Fig.7)

Therefore, the growth curves of these cells in the presence of coated nanoparticles were drawn up exponentially. The factors that can cause the less decrease of magnification coefficient of the fibroblast growth curve equation in the presence of coated nanoparticles is the thickness of the coating on the nanoparticles and the molecular weight of the shell formed on the particles.

CONCLUSIONS

TEM images,VSM, XRD and FTIR results and cell culture showed that the synthesized particles have been Fe_3O_4 and since the saturation magnetization curve of all particles, whether coated with two types of chitosan or uncoated, has crossed the coordinates center, it can be said that there is no residual magnetization, so the resulting particles are super paramagnetic. On the other hand, the thickness of the shell formed on the nanoparticle and also the molecular weight of the chitosan has a direct effect on the amount of saturation magnetization of the super paramagnetic nanoparticles. Therefore, increasing the thickness of the nanoparticle shell can reduce saturation magnetization, and reducing the molecular weight will increase the saturation magnetization of coated nanoparticles. The coating is done in one-stage and perfect, and the cover is surrounded the particle uniformly. Non-coated particles in the medium create much toxicity. However, coating particles with chitosan polymer can reduce the pure particle toxicity. The molecular weight of chitosan can affect the growth of cells over time. So that the particle growth curve with low molecular weight chitosan coating has a smaller magnification factor than particles with medium molecular weight chitosan coatings.

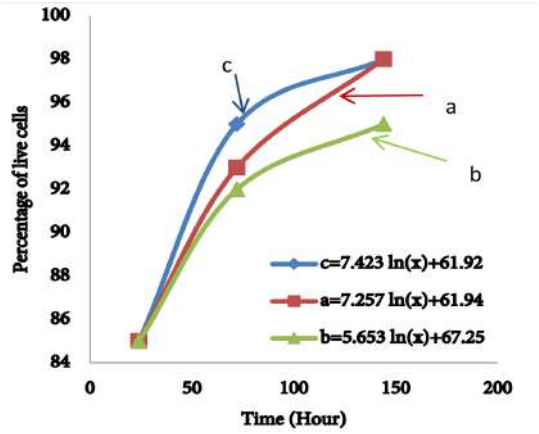

Fig. 7. The growth curve of fibroblast cells in the presence or absence of coated particles. a)Fe_3O_4-Medium molecular weight chitosan, b)Fe_3O_4-Low molecular weight chitosan, c)Control group

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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