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Study of the incorporation of nucleic acids in chitosan-coated polystyrene nanoparticles for use as DNA carrier system.

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Abstract. Nanotechnology is a multidisciplinary scientific field based on the development, characterization, production and application of structures, devices and systems with shape and size at the nanoscale. Polymeric systems with therapeutic purpose have been widely used since they allow a slow and gradual release of drug and allow the transport of drugs to their specific place of action. In recent years, nanoparticles have been used for DNA loading. The introduction of exogenous DNA into a cell may be applicable to fields of gene therapy, DNA vaccines and diagnosis. The development of nucleic acid loading nanoparticles, with a well characterized activity, would be very important. For this project, cationic polystyrene nanoparticles coated with chitosan was studied for a DNA carrier system. The propose is an elaboration of a dilution gradient that allows to know the pattern of incorporation of nucleic acids in the nanoparticles, permitting the development of a mathematical model that characterizes the incorporation in the different conditions studied, allowing their use in future projects. Through this, it´s found the potential of DNA saturation by this nanoparticle system, as in 29% of the incorporation mass, which reveals the capacity of DNA incorporation.

Keywords. *Polymeric nanoparticles, Nucleic acids, Carriers systems, DNA.*

Introduction. Nanotechnology is a multidisciplinary scientific field based on the development, characterization, production and application of structures, devices and systems with shape and size at the nanoscale [1, 2].

Science and technology at nanoscale is a completely new field, encompassing virtually many areas of science and engineering. In one of its aspects, nanomaterials draw great attention for their unique properties and performance improvements, which are defined by size, surface structure and interparticle interactions [3, 4, 5].

Polymeric systems with therapeutic purpose have been widely used since they allow slow and gradual release of drug and allow the transport of drugs to their specific place of action, a fact that is considered the most important aspect in loading them, evidencing the need for suitable carriers [6].

Polymeric nanoparticles, used in systems for controlled drug release, have been studied since the 1970s, aiming at the transport of substances in organisms, tissues and (or) cells, improving

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therapeutic efficacy and reducing toxic effect of the substances administered [7, 8, 9, 10]. They may be defined as colloidal systems having dimensions between 10 and 1000 nm, which may have the drug dissolved, coated, encapsulated or dispersed [11, 12].

According to Abdelwahed W., et. al. 2006 [13], nanoparticles are classified into two groups, differing in relation to composition and structural organization: nanospheres and nanocapsules. Nanocapsules are vesicular systems in which drug is contained within an aqueous or oily cavity surrounded by a polymeric membrane. Nanospheres are formed by a polymer matrix where the drug is dispersed or adsorbed [13].

In recent years, nanoparticles have been used for DNA loading. The introduction of exogenous DNA into a cell may be applicable to fields of gene therapy, DNA vaccines and diagnosis methods [14, 15, 16, 17], in addition to its applicability related also to genomic DNA analysis in urine for medical and forensic tests [18, 19, 20, 21].

Another use for the incorporation of DNA into nanoparticles would be for extraction of nucleic acid directly from electrophoresis gels. The electrophoresis gel technique is widely used for isolation and purification of specific DNA fragments, and their purified fragments can be used for, for example, for molecular cloning [22, 23, 24, 25]. Therefore nanoparticles that could extract DNA from electrophoresis gels for later use would be of great interest to laboratories working, for example, cloning technics [26, 27, 28].

In other researches of our department (Pharmacological Sciences – USP), molecular cloning technology is a very important technic, used in several works. The development of nucleic acid loading nanoparticles, with a well characterized activity, would be very important. For this project, in collaboration with research group of Denise F. S. Petri, PhD (Chemistry Institute – USP), cationic polystyrene nanoparticles coated with chitosan, which had been studied in other applications [27], with indications of being a possible and promising nucleic acid carrier. This project proposes elaboration of a dilution gradient that allows to know the pattern of incorporation of nucleic acids in the nanoparticles, permitting the development of a mathematical model that characterizes the incorporation in the different conditions studied, allowing their use in future projects.

Materials and methods. The collection of nucleic acid samples, for nanoparticles incorporation, starts with RNA extraction and cDNA production. Total RNA was obtained from cell culture, of HUVEC (Human umbilical vein endothelial cell) and HEPG2 (Liver hepatocellular cells) lineages. Cells were cultured on DEMEM medium adhered to the bottle. After confirmation of the characteristic growth pattern under inverted optical microscope in the 20ml of the initial culture, all the medium were removed and added 2 ml of trypsin for 5 minutes to disrupt cells. The cells were then resuspended in 20 ml of culture medium, reaching a final concentration of approximately 2x10^6 cells per ml of culture medium, then incubated 2ml of culture in each well of a 6-well plate for 24 hours.

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Next, the extraction of total RNA was made using 1 ml of Trisol to make 2 ml of culture, 200 µl of chloroform, centrifuging at 12000G for 14 minutes. Transferring clear phase to another 1.5ml tube with 500µl of isopropanol. Precipitating on dry ice for 45 minutes and centrifuge again under the same conditions described above. We discarded supernatant, and resuspended in 1ml of 70% ethanol, centrifugation at 7500G for 7 minutes. Finally, discarded supernatant and resuspended the pallet in 50 µl of deionized water.

The cDNA was obtained by reverse transcription from total mRNA, performed in vitro. For reaction, we used 1µL Reverse Transcriptase enzyme and 4µl buffer, 1µl of random primers, 1µl dNTP, 2µl DTT and 12µl deionized water and 1µl RNA, for a final volume of 20 µl of reaction.

In Verity thermocycler was programmed the first hybridization step 25°C for 10 minutes, then 42°C for 50 minutes for enzyme action, and finally 70°C for 15 minutes for inactivation of enzyme.

The Study of incorporation of nucleic acids into chitosan-coated polystyrene nanoparticles were assigned by Prof. Dr. Denise F. S. Petri of Institute of Chemistry of the University of São Paulo, being made, characterized and sterilized according to Castro L. B. R., et. al. 2007 [27].

A 0.8% agarose gel was prepared to perform electrophoresis, to confirm the aggregation of DNA to nanoparticles. The control samples were made with isolated DNA (3µl of DNA and 5µl of sterile deionized water) and isolated nanoparticles (3µl of sterile deionized water and 5µl of nanoparticles). The sample was prepared mixing 3µl of total DNA and 5µl of nanoparticles, which was kept under stirring for 1 minute, then centrifuged at 13000 rpm for 5 minutes to separate precipitate supernatant. After that, samples were applied to gel for electrophoresis. In all samples were added with 1µl Gel Red marker and 1µl run buffer, and the nanoparticles were at a concentration of 3.3 x10^12 particles / L. The gel image was obtained by transluminator, in an exposure time of 2.74 seconds.

Gradient dilutions were performed, with sterile deionized water, on nanoparticles to establish a lower concentration. Due to the fact that nanoparticles were initially at a concentration of 3.30 x 10^12 particles / L, a concentration was chosen for the study of 1.24 x 10^12 particles / L in order to achieve a greater amount of material for the study. Such concentration showed good capacity for DNA incorporation. All samples were quantified by spectrophotometry (ND-1000, NanoDrop), for the elaboration of dilution gradient.

A pH gradient (6.0-8.0) was studied to verify the most favorable environment for incorporation of DNA into nanoparticles through Tris-HCl buffer solutions, performed in triplicate and analyzed for related uncertainties by the standard deviation of the mean.

The best pH for incorporation was defined, a final volume of 30µl was fixed, 2µl of nanoparticles were added to the DNA samples, maintained at a concentration gradient of 100-250ng / µl, followed by shaking for 1 minute to ensure full adherence of the material. All samples were centrifuged at 13000 rpm for 5 minutes for precipitation of nanoparticle clusters with DNA, and measurements of supernatant were immediately measured to verify that the excess DNA was not incorporated.

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Results and discussion. The Collection of nucleic acid samples for incorporation with RNA extraction and cDNA extraction occurred efficiently. An approximate concentration of 200ng / µl, quantified in a spectrophotometer (ND-1000, NanoDrop) with absorbance in the range 260nm. This allowed the elaboration of gradient stipulated in the project, without the necessity of concentration of the samples.

The study of incorporation of nucleic acids in nanoparticles starts with Electrophoresis, performed at 100V and 150mA for 20 minutes, as in the following result, presented in Fig. 1:

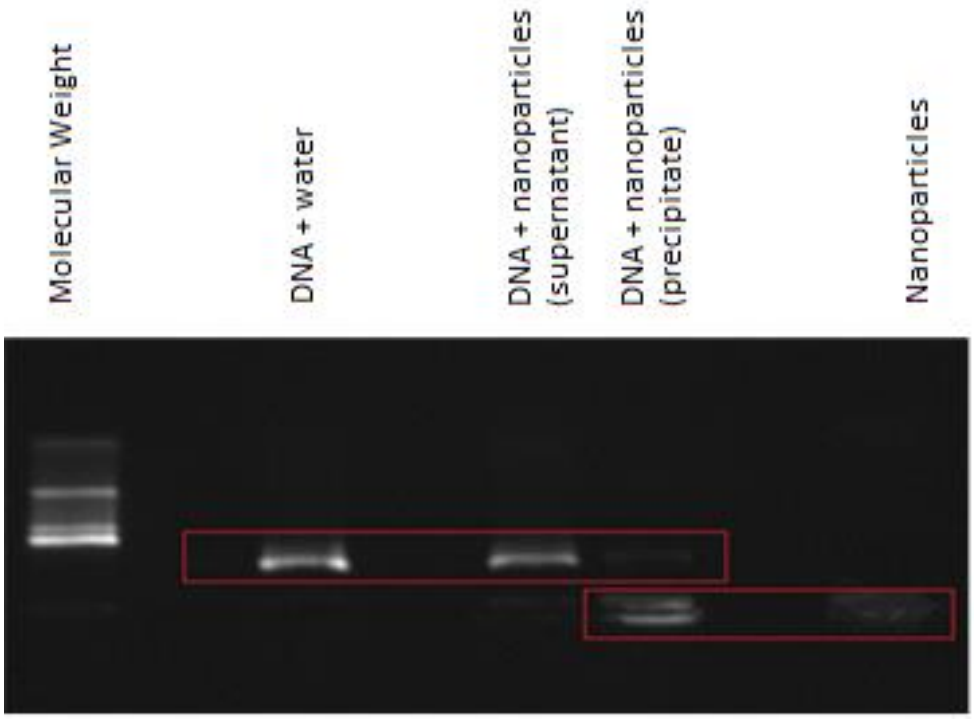

Figure 1. Comparative electrophoresis gel between supernatant and mixture precipitate Total DNA + nanoparticles.

Figure 1 shows that nanoparticles alone presents a certain degree of fluorescence when submitted to UV radiation (last column of the gel), but the fluorescence presented by portion of the precipitate has a considerably higher intensity, thus proving the incorporation of the total DNA by nanoparticles (column DNA + nanoparticles precipitated).

Regarding the study to define the best pH for the incorporation, we were able to produce the following graph, in Fig. 2:

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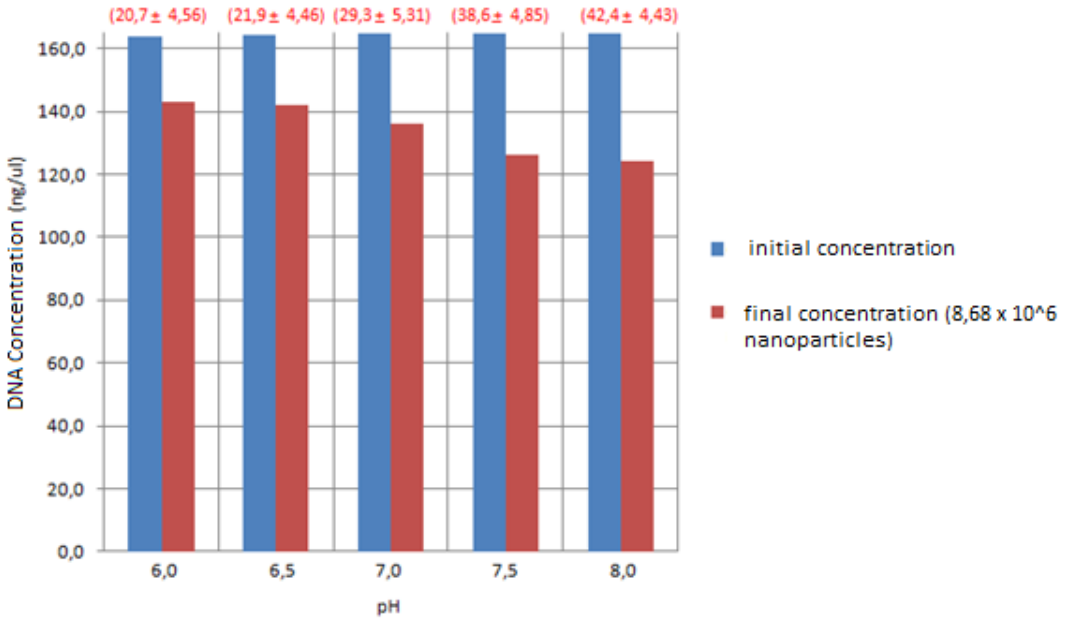

Figure 2. Graph pH x total DNA concentration, with deltas explained above the bars with their respective uncertainties.

With these results, it is possible to verify a better incorporation in more basic pHs. Although pH 7.5 or 8.0 not guaranteed the best incorporation, because its limits of uncertainty define an overlap of values of variation of concentration, so were defined pH 8.0 for carrying out all subsequent experiments.

As a result of the studies of incorporation of total DNA, after all the steps of agitation, centrifugation and quantification of the supernatant we were able to make the following chart, Fig. 3. This graph allows us to realize that, at high concentrations of total DNA, the incorporation takes place in a more accentuated way, fact that becomes clearer with the analysis of following Table 1.

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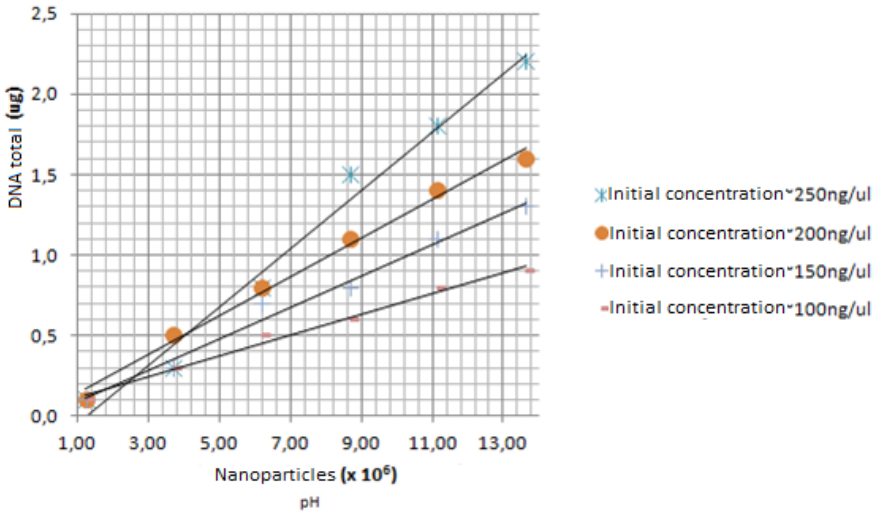

Figure 3. Graph of the relationship between amount of total DNA and amount of nanoparticles.

Table 1. Parameters of the lines of Figure 3.

Total DNA initial concentration [ng/ul]	Linear correlation	line equation	Angular coefitient (x 10^-6)
250,0	0,9813	y = 0,1809x - 0,229	0,16
200,0	0,9916	y = 0,121x + 0,0167	0,12
150,0	0,9846	y = 0,0979x - 0,0119	0,10
100,0	0,9882	y = 0,0645x + 0,0533	0,07

With the presentation of Table 1, the high correlation between the drawn line and the data obtained experimentally, and the greater intensity of incorporation in higher concentrations of total DNA can be evidenced. In the presented equations, "y" refers to the amount in µg of total DNA incorporated and "x" to the amount of nanoparticles multiplied by 10^6, representing the mathematical model of this system.

Conclusion. The use of chitosan coated polystyrene nanoparticles in the incorporation of DNA molecules is proven. The mathematical model is a linear straight line method and its angular coefficient variation. It is possible that the straight line dilution rates reveal a progressive amount of free DNA, for each free DNA condition in which nanoparticles are submerged. Through this, it´s found the potential of DNA saturation by nanoparticles, as in 29% of the incorporation mass, which reveals the potential of DNA incorporation of the system. Therefore, the experiment was effective, reproducible and promising to nanoparticles system, which can be used as DNA carriers.

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