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Full Paper

The Cytotoxicity, Characteristics, and Optimization of Insulin-loaded Nanoparticles

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Abstract: Controlled release systems for insulin are frequent subjects of research, because it is rapidly degraded by proteolytic enzymes in the gastrointestinal tract and minimally absorbed after oral administration. Controlled release systems also provide significant contribution to its stability. Different techniques are used for the preparation of drug-loaded nanoparticles, and many novel techniques are being developed. The size and morphology of insulin-loaded nanoparticles may vary according to performed techniques, even if the same polymer is used. The aim of this study was to demonstrate the cytotoxicity of insulin loaded nanoparticles and the effect of various synthesis parameters on the particle size, polydispersity index (PdI), loading efficiency, and particle morphology. In the experiments, poly(lactic-co-glycolic acid) (PLGA) and insulin-loaded PLGA nanoparticles were prepared using the double emulsion (w/o/w) method. The characterization of the nanoparticles were performed with a UV spectrometer, the Zeta-sizer system, FTIR spectroscopy, and a scanning probe microscope. Cell toxicity of different concentrations was assayed with MTT methods on L929 fibroblast cells. The optimum size of the insulin-loaded PLGA nanoparticle was obtained with a 96.5% encapsulation efficiency, a 224.5 nm average particle size, and a 0.063 polydispersity index. This study obtained and characterized spherical morphology, determined that the nanoparticles have very low toxicity, and showed the effect of different parameters on particle size and polydispersity.

Keywords: cell culture; controlled release system; cytotoxicity; insulin

1. INTRODUCTION

Insulin plays a central role in glucose metabolism in the body [1] and is composed of two polypeptide chains called the A and B chains [2]. It is the most effective drug in the treatment of diabetes mellitus for adjusting glucose levels [3, 4]. Insulin administration is applied subcutaneously, since it is rapidly degraded by proteolytic enzymes in the gastrointestinal tract and slightly absorbed after oral administration [5]. However, oral administration would avoid the potential for contamination, pain, and discomfort of injections [6].

Recently, polymeric nanoparticles have gained importance in pharmaceutical applications due to their important contribution to the stability of proteins in the gastrointestinal tract and facilitation of the oral use of proteins. The encapsulation of different molecules has extended the number of the fields to which nanoparticles can be applied [7, 8] and it allows the modulation of physicochemical characteristics, drug release properties, and biological behaviors [6]. Polymeric nanoparticles are mainly used as drug delivery vehicles because of their properties of biodegradability, high encapsulation ability, controlled release, and low toxicity [9].

Insulin is also encapsulated in biodegradable nanostructures, such as nanocapsules, nanospheres, or microspheres [10]. New generation nanocarriers for insulin delivery are poly(lactic-co-glycolic acid) (PLGA), dextran, solid lipid, and polyalkylcyanoacrylated-insulin nanoparticles [11]. However, even if the same polymer is used in different studies, because of the different techniques employed, the size and morphology of insulin-loaded nanoparticles may vary. In one study, the size of insulin-loaded nanoparticles was generally in the range of 100-300 nm[12]. In another study conducted by Cui et al. [13], the size of PLGA-based insulin nanoparticles were reported to be in the range of 146-201 nm (10th to 90th percentile), spherical in shape,

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and negatively charge. In a different study, PLGA-polyvinyl alcohol (PVA) nanoparticles for insulin delivery that were targeted and spherical, 161 nm in size, and homogeneous (polydispersity index 0.082), and negatively charged were obtained [14].

There are still different techniques for preparation of drug-loaded nanoparticles, and many novel techniques are being developed [15]. As seen in the literature, the method strongly affects the size of insulin-loaded PLGA nanoparticles. Therefore, the aim of this study was to illustrate the cytotoxicity of insulin-loaded nanoparticles which synthesized with various parameters (sonication power, time, etc.) and determine the effect of these parameters on the size and polydispersity index, loading efficiency, and particle morphology by using a Zeta-sizer, scanning probe microscope, and Fourier transform infrared (FT-IR) spectroscopy.

2. MATERIAL AND METHODS

Human insulin (M_w ~ 5800 Da), PLGA (lactide:glicolide = 50:50, M_w ~ 38-54 kDa), 3-(4,5-dimethyl thiazole-2-yl)-2,5-diphenyltetrazoliumbromide (MTT), dimethyl sulfoxide (DMSO), and (PVA) were purchased from Sigma-Aldrich (St. Louis, USA). All the chemicals and solvents were of analytical grade and used without further purification. Ultra-pure water was obtained using the Millipore MilliQ Gradient System.

2.1. Nanoparticle Preparation Method

PLGA and insulin-loaded PLGA nanoparticles were synthesized with the double emulsion method (water-in-oil-in-water or w/o/w). PLGA was dissolved in dichloromethane (DCM), and insulin dissolved in water was added. This water/oil emulsion was homogenized using a sonicator (Bandelin, Germany) in an ice bath and with different values of energy output and time. Following this step, a PVA aqueous solution was added to support particle shape, and then the emulsification step with the sonicator was repeated. Then, emulsions were stirred using a magnetic stirrer at room temperature for 16 hours to evaporate the organic solvent, thereby reducing toxicity. Nanoparticles were centrifuged for three times at 10,000 rpm for 20 minutes (Hettich, Germany), washed three times with ultra-pure water to remove the impurities, and then characterized.

2.2. Determination of Average Size, Polydispersity, and Size Distribution with Dynamic Light Scattering

Photon correlation spectroscopy with a Zeta-sizer Nano ZS (Malvern Instruments, UK) instrument, equipped with a 4.0 mV He-Ne laser (633 nm) at a temperature of 25°C, was carried out to examine the properties of the average size, PDI, and size distribution of insulin-loaded PLGA nanoparticles. Before every electrophoretic light scattering measurement, each sample was prepared with phosphate-buffered saline (PBS) and filtered with 0.2 µm regenerated cellulose membrane (Sartorius, Germany) filters to remove the impurities from the solutions.

2.3. Determination of the Encapsulation Efficiency of Insulin

The encapsulation efficiency of insulin was detected by using ultraviolet visible (UV-Vis) spectroscopy at 280 nm. Primarily, the standard calibration curve of insulin was obtained. After the centrifugation step of insulin-loaded PLGA nanoparticles, the encapsulation efficiency of insulin was determined by measuring the concentration of free insulin in the supernatant. This was determined from the standard calibration curve of insulin.

$$Encapsulation\ Efficiency = \frac{Total\ insulin\ amount - Free\ insulin\ amount}{Total\ insulin\ amount} \times 100$$

2.4. Fourier Transform Infrared (FT-IR) Spectroscopy

An IR Prestige21 FT-IR spectrophotometer (Shimadzu, Japan) was used for chemical analyses of the functional groups present in the samples. Measurements were carried out for PLGA, PLGA nanoparticles, and insulin-loaded PLGA nanoparticles in universal attenuation total reflectance (ATR) mode. The FT-IR spectra were obtained with four scans per sample ranging from 4,000 to 750 cm^{-1} and a resolution of 4 cm^{-1}.

2.5. Obtaining Images of Nanoparticles with the Scanning Probe Microscope

The optimized insulin-loaded PLGA nanoparticles were analyzed with a scanning probe microscope (Shimadzu Scanning Probe Microscope SPM-9600) in "Dynamic Mode." In this procedure, a silicium SPM sensor (Nanoworld, NCHR, Non-contact Mode) was used; this is compatible with "Dynamic Mode." Five microliters of sample solution were dropped onto the mica surface. After 5 minutes, the solution was diluted with ultra-pure H_2O to remove the surface. Twenty minutes later, after drying the mica, the insulin-loaded PLGA nanoparticles were imaged with SPM.

2.6. Cytotoxicity Experiments

The L929 cell line was used for the cytotoxicity studies. Cells were cultured in a DMEM-F12 medium (supplemented with 10% fetal bovine serum and penicillium-streptomycin) and incubated at 37°C for 3 days until they were confluent in the 5% CO_2 incubator. A trypsinization process was applied on the cells, and detached cells were obtained with centrifugation.

L929 cells with a concentration of 10^5 cell/mL were seeded into a 96 well plate. After 24 hours of incubation, different concentration of nanoparticles were added to the wells and incubated for 24 hours. After that MTT solution was prepared and loaded into the wells. It was incubated for 4 hours in a dark environment at 37 °C. Following this, liquids containing MTT solution were aspirated and 100 µL DMSO was added to each well at room temperature. After incubation for 30 minutes, the optical density was measured at 570 nm with multiplate reader. Then, the percent viability was calculated with the equation:

$$\%\text{Viability} = \frac{\text{Absorbance of experimental group}}{\text{Absorbance of control group}} \text{x}100$$

3. RESULTS AND DISCUSSION

Insulin is a protein hormone that is easily degraded in the gastric environment. According to literature of recent years, numerous investigations based on different release systems have aiming to develop a more efficient delivery of insulin. Both hydrophilic and hydrophobic polymers have been used for the controlled release of insulin. However, according to literature analysis, the physicochemical characterizations of the evaluated nanoparticle formulations has not always been adequate [16]. In this study, a biodegradable PLGA polymer with Food and Drug Administration (FDA) approval was selected for the controlled release of insulin. PLGA has been selected by various researchers for the same aim, but the optimization method is another important issue.

PLGA and insulin-loaded PLGA nanoparticles were prepared using the double emulsion method, which was carried out for different values of energy output and time. The Zeta-sizer results are given in Table 1 with the values of particle size and PdI. These results indicate that sample prepared by third method has narrow size distribution with 0.063 of polydispersity index and 224.5 nm of minimum particle size.

Table 1. Optimization parameters of the nanoparticle preparation method.

Samples	Sonicator Energy (Watt)	Minute (w/o)	Minute (w/o/w)	Particle Size (nm)	Polydispersity Index (PdI)
1	60	5	5	255.6	0.063
2	60	4	4	283.8	0.14
3	50	5	5	224.5	0.063
4	50	4	4	305.2	0.3
5	70	4	4	283.6	0.095
6	70	5	5	320.4	0.35

For the calculation of insulin encapsulation efficiency, the standard calibration curve of insulin was formed with different insulin concentrations, as shown in Figure 1. By analyzing the supernatants of

nanoparticles with UV spectroscopy at 280 nm, insulin concentrations in the supernatants were determined and the encapsulation efficiency of the insulin nanoparticles was calculated by the formula shown above. The results are given in Table 2 and these results indicate that the encapsulation efficiency of insulin nanoparticles has highest value with 96.5% in third method.

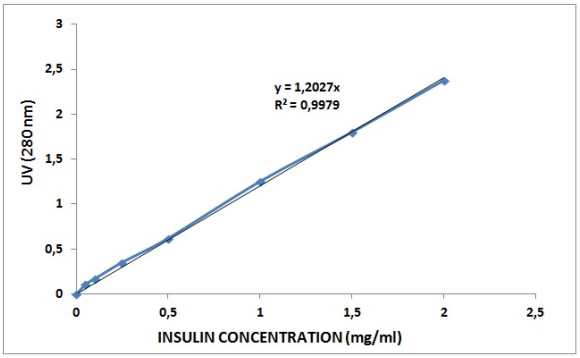

Figure 1. The standard calibration curve of insulin at 280 nm.

Table 2. Encapsulation efficiency of insulin-loaded nanoparticles.

Sample	UV_{280nm}	Free Insulin Amount (mg/mL)	Encapsulation Efficiency
1	0.101	0.084	91.6
2	0.6122	0.509	49.1
3	0.042	0.0349	96.5
4	0.3034	0.2523	74.77
5	0.4796	0.3988	60.12
6	0.333	0.2768	72.32

The FTIR spectra of PLGA, PLGA nanoparticles, and insulin-loaded PLGA nanoparticles are shown in Figure 2. The absorption bands at 2994 and 2946 cm^{-1} were the C-H stretch of CH_2 and the C–H stretch of –C–H–. A strong band at 1770 cm^{-1} was attributed to the stretching vibration of the C=O of the ester bond. The typical peaks of the PLGA molecule, 1180 and 1070 cm^{-1}, were assigned to C–O stretching (Table 3). When we compared three FTIR spectra of PLGA, as seen in Figure 2c, the peaks at 1770, 1180, and 1070 cm^{-1} increased dramatically in the spectra of insulin-loaded PLGA nanoparticles. The intensity increases in these peaks are clearly an affirmation of insulin encapsulation in PLGA.

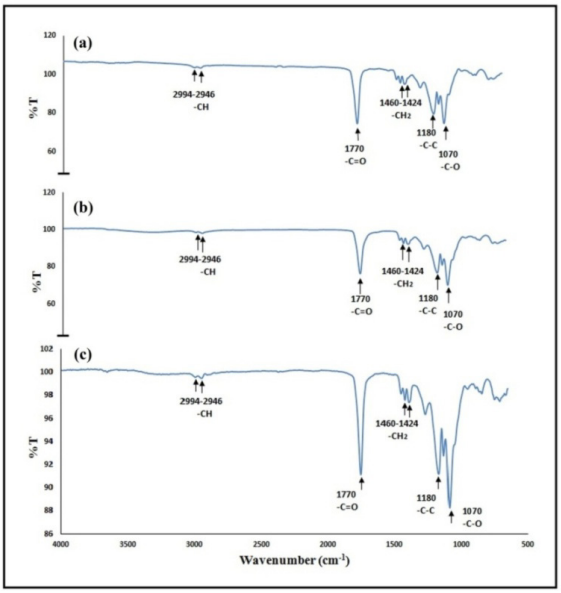

Figure 2. FT-IR spectrum of PLGA **(a)**, PLGA nanoparticles **(b),** and insulin-loaded PLGA nanoparticles **(c)**.

Table 3. Functional groups of PLGA.

Functional Groups	Wavenumber (cm^{-1})
-C – O	1070
-C – C	1180
$-CH_2$	1424 - 1460
-C = O	1770
-CH	2946 - 2994

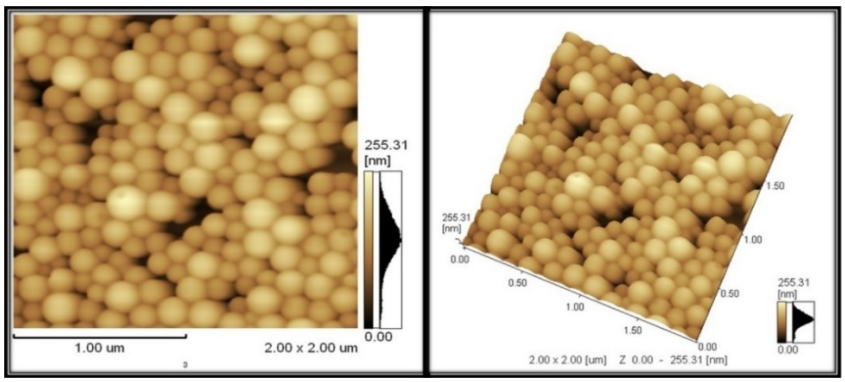

Figure 3. Scanning probe microscopy images of optimized insulin-loaded PLGA nanoparticle.

The spherical and non-aggregated morphology of the optimized insulin-loaded PLGA nanoparticles was shown via SPM images in Figure 3. Therefore, the present study showed different optimization parameters and their effects on particle properties. The obtained 224.5 nm of particle size, 0.063 polydispersity index, and 96.5% of encapsulation efficiency indicate that the third method is optimum.

After the optimization and characterization experiments of the nanoparticles, optimized insulin-loaded PLGA nanoparticles and PLGA nanoparticles were used in six different concentrations (C=1, 5, 25, 50, 250, or 500 µl/mL) in cell culture experiments. The toxicities of PLGA and insulin-loaded PLGA nanoparticles were assayed via the MTT method, and results showed that PLGA and insulin-loaded PLGA nanoparticles have similar toxicity profiles. Figure 4 demonstrates that, even at high concentrations, the investigated particles decreased cell viability by approximately 20% compared to the controls. This showed that neither nanoparticle had high toxicity in fibroblast cells.

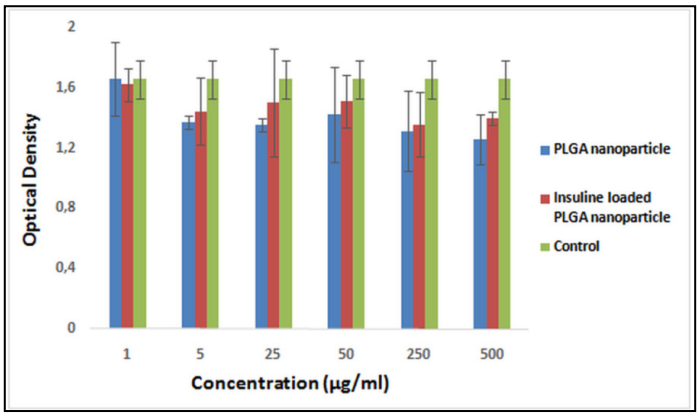

Figure 4. Viability of cells exposed to different concentrations of PLGA and insulin-loaded PLGA nanoparticles.

As a consequence of the cell culture experiments, it was observed that PLGA and insulin-loaded PLGA nanoparticles have no toxic effect in low concentrations. At high nanoparticle concentrations, the viability was found to be around 80%.

4. CONCLUSION

This study demonstrated the nanoparticles' low toxicity, obtained and characterized spherical morphology, and showed the effect of different parameters on particle size and polydispersity. This methodology may be used by various researchers in the future for different protein-based drugs and vaccines. Future studies should focus on determining the in vivo efficiency of obtained insulin-loaded particles in animal models.

5. ACKNOWLEDMENTS

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6. REFERENCES AND NOTES

[1] Insel, P.A.; Liljenquist, J. E.; Tobin, J. D.; Sherwin, R. S.; Watkins, P; Andres, R.; Berman M. *J. Clin. Invest.* **1975**, *55*, 1057. [CrossRef]

[2] Goeddel, D. V.; Kleid, D. G.; Bolivar, F; Heyneker, H. L.; Yansura, D. G.; Crea, R.; Hirose, T.; Kraszewski, A.; Itakura, K.; Riggs, A.D. *Proc. Natl. Acad. Sci.* **1979**, *76,* 106. [CrossRef]

[3] Perez-Nieves, M.; Kabul, S.; Desai, U.; Ivanova, J. I.; Kirson, N. Y.; Cummings, A. K.; Birnbaum, H. G.; Duan, R.; Cao, D.; Hadjiyianni, I. *Curr. Med. Res. Opin.* **2016**, *32,* 669. [CrossRef]

[4] Samsom, M.; Trivedi, T.; Orekoya, O.; Vyas, S. Oral health Case Rep. **2016**, *2,* 112.

[5] Shinichiro, H.; Takatsuka, Y.; Hiroyuki, M. *Int. J. Pharm.* **1981**, *9,* 173. [CrossRef]

[6] des Rieux, A.; Fievez, V.; Garinot, M.; Schneider, Y. J.; Préat, V. *J. Control. Release* **2006,** *116,* 1. [CrossRef]

[7] Singh, M.; Manikandan, S.; Kumaraguru, A. K. *Res. J. Nanosci. Nanotechnol*. **2011**, *1,* 1. [CrossRef]

[8] Wang, F.; Chen, Y.; Benson, H. *Open Drug. Deliv. J*. **2008**, *2*, 1.

[9] Kumari, A.; Yadav, S. K.; Yadav, S. C. *Colloids Surf. B: Biointerfaces* **2010**, *75*, 1. [CrossRef]

[10] Kumar, C. S. *Nanotechnologies for the Life Sciences.* Vol. 10.: John Wiley & Sons, 2007. [CrossRef]

[11] Sharma, G.; Sharma, A. R.; Nam, J. S.; Doss, G. C.; Lee, S. S.; Chakraborty, C. *J. Nanobiotech*. **2015,** *13,* 1. [CrossRef]

[12] Zhang, L.; Ling, L.; Zhou, L.Y.; Wu, Z.M.; Guo, X.D.; Jiang, W.; Luo, Q.; Qian, Y. pH-sensitive nanoparticles for oral insulin delivery. 2014, Google Patents.

[13] Cui, F.; Shi, K.; Zhang, L.; Tao, A.; Kawashima, Y. *J. Control. Release* **2006**, *114,* 242. [CrossRef]

[14] Sun, S.; Liang, N.; Piao, H.; Yamamoto, H.; Kawashima, Y.; Cui, Fude. *J. Microencapsul*. **2010**, *27,* 471. [CrossRef]

[15] Bala, I.; Hariharan, S.; Kumar, M. N. *Crit. Rev. Ther. Drug Carrier Syst*. **2004**, *21,* 387. [CrossRef]

[16] Card, J. W.; Magnuson, B. A. *Am. J. Physiol*. **2011**, *301,* G956-67. [CrossRef]