



The effect of synthesized chitosan grafted poly (N-L-lactide) on human genetic material

Efecto del injerto poli-quitosano (N-L-lactide) sintetizado sobre el material genético humano

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SCIENTIFIC RESEARCH

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ABSTRACT

Introduction: Chitosan is one of the natural polymers can generally consider as a biocompatible and biodegradable polycationic polymer, which has minimum immunogenicity and low cytotoxicity. Therefore, chitosan and its derivatives may represent potentially safe cationic carriers for use in gene delivery. **Materials and Methods:** Chitosan with 90.1 DD% obtained by deacetylation of chitin extracted from local shrimp shells. Graft copolymerization of L-lactide onto chitosan was carried out at room temperature by ring opening polymerization under a nitrogen atmosphere to prepare chitosan-g-poly (N-lactide) graft copolymer. It was obtained in good yield and characterized by FTIR. The samples purity and concentration were detected using both Nanodrop UV-spectroscopy and agarose gel electrophoresis techniques. The human heat shock proteins gene, *hsp-70*, was used as a model of human genes to study the effect of chitosan-g-poly (N-lactide) graft copolymer. **Results and Discussion:** The results revealed that chitosan-g-poly (N-lactide) graft copolymers had safety effect on the DNA, and binding with it. the human heat shock proteins gene, *hsp-70*, was used as a model of human genes to study the effect of chitosan-g-poly (N-lactide) graft copolymer, it shows a good binding ability the human gene, implies that it might be used in biomedical applications in the future. **Conclusions:** Grafting of L-lactide onto chitosan by ring opening polymerization was confirmed by FTIR. The prepared polymer has safety effects on human DNA and genes. The chitosan-g-poly (N-lactide) graft copolymer has shown highly efficiency to electrostatic interaction with human DNA and gene, implying that it is suitable to be used as DNA and gene delivery.

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INTRODUCTION

Chitosan is a deacetylated derivative of chitin (N-acetyl-2-amino-2-deoxy-D-glucopyranose units, linked by β -D (1-4) bonds ⁽¹⁾. These units are distributed randomly or in block throughout the polymer chain depending on the processing method used to obtain the biopolymer derivatives ^(2,3). The chitosan is characterized by its acetylation degree (AD) or deacetylation (DD) ⁽⁴⁾, which is calculated statistically by the proportion of D-glucosamine and N-acetyl-D-glucosamine by its molecular weight ⁽⁵⁾. The chitosan amino group has a pKa value of ~ 6.5 , so, chitosan can be positively charged and soluble in acidic to neutral solution with a charge density practically depends on pH and degree of deacetylation percentage ⁽⁶⁾.

Chitosan is one of that can generally consider as biocompatible and biodegradable polycationic polymer, which has minimum immunogenicity and low cytotoxicity ⁽⁷⁾. Therefore, chitosan and its derivatives may represent potentially safe cationic carriers for use in gene delivery ⁽⁸⁾. Factors include; degree of deacetylation, molecular weight, and charge of chitosan, and pH media, are significant role in the determination of the transfection efficiency of polyplexes containing chitosan and DNA ⁽⁹⁾.

It's well-known that poly(L-lactide) is a hydrophobic polymer because it has the CH_3 side groups ⁽¹⁰⁾, and it has been widely used in biomedical applications, such as sustained drug delivery systems due to its faster degradation rate ⁽¹¹⁾. Yan Wu and et al., recently reported that poly(L-lactide) is a kind of biodegradable materials with low toxicity, the excellent biocompatibility and bio-absorbability in vivo. In

addition, they were also prepared microcells of chitosan-poly lactide graft copolymer with amphiphilic behavior used as a promising delivery carrier for the entrapment and controlled release of hydrophobic

drugs ⁽¹²⁾. Others were found these micelles with lower initial drug load exhibited a faster release rate ⁽¹³⁾.

Grafting L-lactide onto chitosan is a key for increasing hydrophilicity and controlling degradation rate, and it was achieved by ring opening polymerization using $\text{Ti}(\text{O}i\text{Bu})_4$ as catalysts ⁽¹⁴⁾. Attempting to find other metal catalysts for initiate polymerization process was successfully discovered by Ji Young Kim and et al., using LiCl as a catalyst, high molecular weight of grafting copolymer was obtained ⁽¹⁵⁾. Self-catalysis strategy can be also used for this purpose ⁽¹⁶⁾.

Some reported kind of literature ^(17,18) mentioned that chitosan can be grafted with the terminally functional PLA via the reaction of amino groups. The amino group at is expected to be more reactive than the hydroxyl groups due possibly to their high nucleophilicity. But at the same time it is not possible to totally neglect the reactivity of $-\text{OH}$ groups because this was the main co-initiator used by several research teams in the ROP of lactides. So, there is a possibility that esterification might also take place partly, and the main product being the amide ⁽¹⁹⁾.

In this study, a powerful synthetic strategy for grafting of L-Lactide into chitosan by the ring-opening copolymerization was placed on. Using DUB as an active and safe organic catalyst can provide an alternative method to prepare new chitosan-based biohybrids without toxic metals that may have wide applications in biotechnological and in controlled drug delivery systems.

MATERIALS AND METHOD

MATERIALS

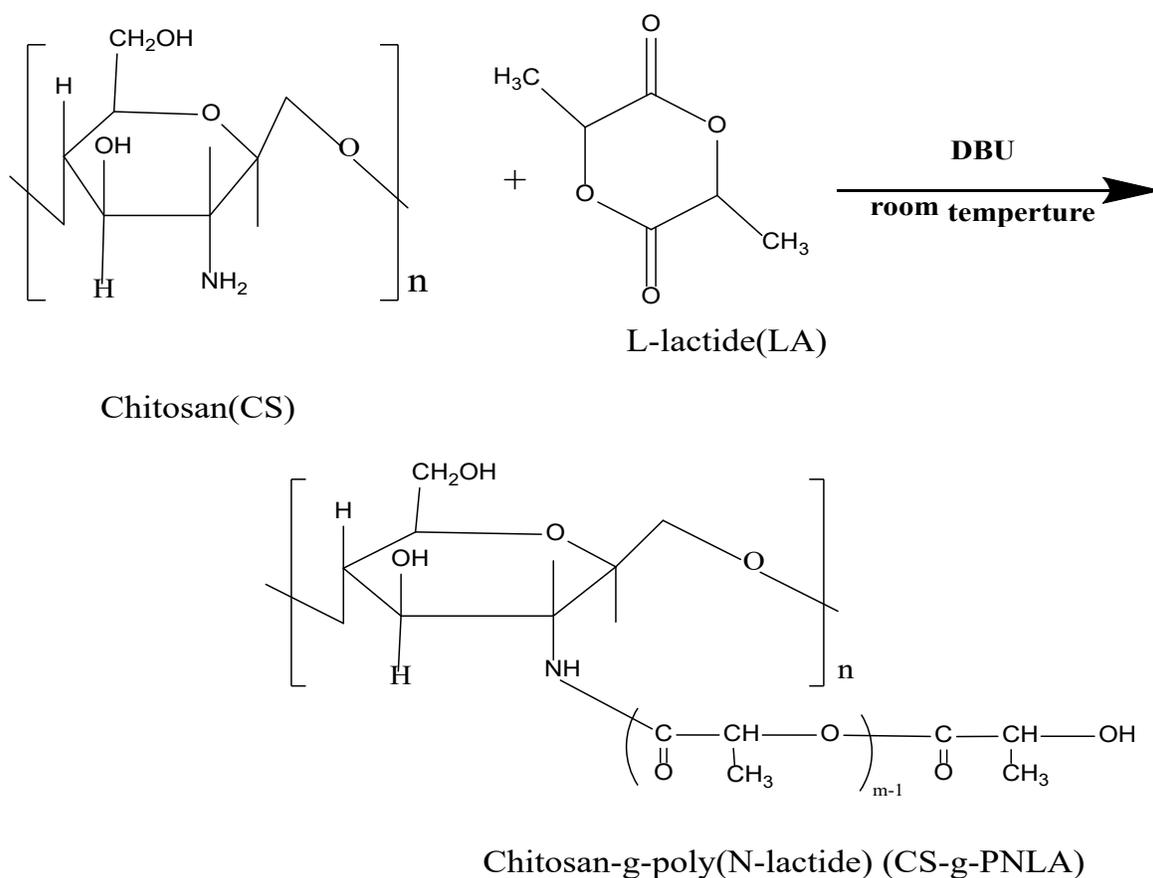
Chitosan with 90.1 DD% obtained by deacetylation of chitin extracted from local shrimp shells as reported in the literature. The extraction process involved drying and grinding the shrimp shells, and

then washed vigorously with (5%) hydrochloric acid solution by mechanical stirrer to remove minerals and their salts to get chitin. This was added to (50%) sodium hydroxide solution, and the mixture was left reflux for 3 hours. Finally, residual was washed with water and dried (20). The product, chitosan, was purified by dissolving in 1% acetic acid and stirred until a homogenous solution is obtained and any insoluble may present, were removed by filtration. Chitosan solution was titrated with 4N NaOH until pH value of 8.5 was reached. The chitosan obtained is washed several times with distilled water, filtered and dried (21).

The L-lactide and 1,8-Diazabicyclo[5.4.0]undec-7-ene, (DBU), were supplied by Sigma-Aldrich. Solvents, toluene and acetone, were also supplied by Sigma-Aldrich and they purified and dried to be used as a reagent for ring-opening copolymerization (22).

METHODS

1g of L-lactide, 1g of purified chitosan and 100 ml of dry toluene were charged in a three-neck round bottom flask fitted with mechanical stirrer, inlet and outlet gas tube. The reaction was carried out in presence of 90 μ l of DBU as a catalyst with stirring for 24 hours at room temperature under dry nitrogen gas. The mixture was filtered and washed by acetone for twice. Next, vacuum was used to dry sample for 24 hours which finally characterized by FTIR. IR spectra were recorded on Fourier-transform infrared spectrometer (JASCO FTIR 4200/Polymer Research Center, University of Basrah)). Chitosan and its graft copolymer were examined as KBr pressed disc. Scheme 1 shows the schematic chemical reaction equation.



Scheme 1. Ring-opening grafting copolymerization of L-Lactide onto chitosan

BIOTECHNOLOGY STUDY

DNA EXTRACTION

Human genomic DNA was extracted using the DNA extraction Mini Kit, (Geneaid Company) from fresh blood samples. The quantity and quality of purified DNA were determined by the Nanodrop (Nanodrop/Optizen, Department of Biology/University of Basrah), at 260/280 nm and agarose gel electrophoresis, respectively, reported as a literature method ⁽²⁰⁾.

EFFECT CHITOSAN-G-POLY(N-LACTIDE) ON HUMAN GENOMIC DNA

Different concentrations chitosan-g-poly(N-lactide) graft copolymers were examined for interaction with DNA and the results were investigated by electrophoresis apparatus (Fisher Scientific/Department of Biology/University of Basrah) using agarose gel. Complexes were prepared by mixing 10 μ l of DNA with 10 μ l of polymer solutions which are: (0.1, 0.4, 0.6, 0.8 and 1) mg/ml (2% HAC). After incubation for 1h at 37°C, the mixture was examined using 0.8% agarose gel; the free human DNA was used as a positive control sample. The gel was run at 60 V for 1 h using 1X TBE buffer as a gel running buffer, after which the bands were visualized using a UV light transilluminator (ATTA, Department of Biology/University of Basrah). The concentration of DNA and absorption were determined by UV spectroscopy at 260 nm, to determine the percentage of the binding nucleotide in DNA samples with polymers.

The mixtures were undergoing electrophoresis for 1h on a 0.8% agarose gel in. Ethidium bromide was used to stain the gel and photographed under UV transilluminator.

DETECTION OF THE HEAT SHOCK PROTEIN GENE BY POLYMERASE CHAIN REACTION (PCR)

The amplification method using specific sequences of primers according to the Kowalczyk et al. ⁽²³⁾ for detecting the heat shock protein-70 genes (*hsp-70*) was used and the amplified PCR (Eppendorf/Department of Biology/University of Basrah) products were electrophoresed using 2% agarose gel ethidium bromide-stained.

STUDY THE BINDING AFFINITY OF CHITOSAN-G-POLY (N-LACTIDE) WITH HUMAN GENE

10 μ l of heat shock protein gene with 10 μ l of prepared polymer with concentration (1 mg/ml) were mixing then subjected to electrophoresis using 2% agarose, to determine the binding affinity of these polymers with human genes.

RESULTS

FTIR CHARACTERIZATION

FTIR spectroscopy was used to characterized the extracted chitosan and the prepared chitosan-grafted-poly (N-lactide) copolymer, Figure 1.

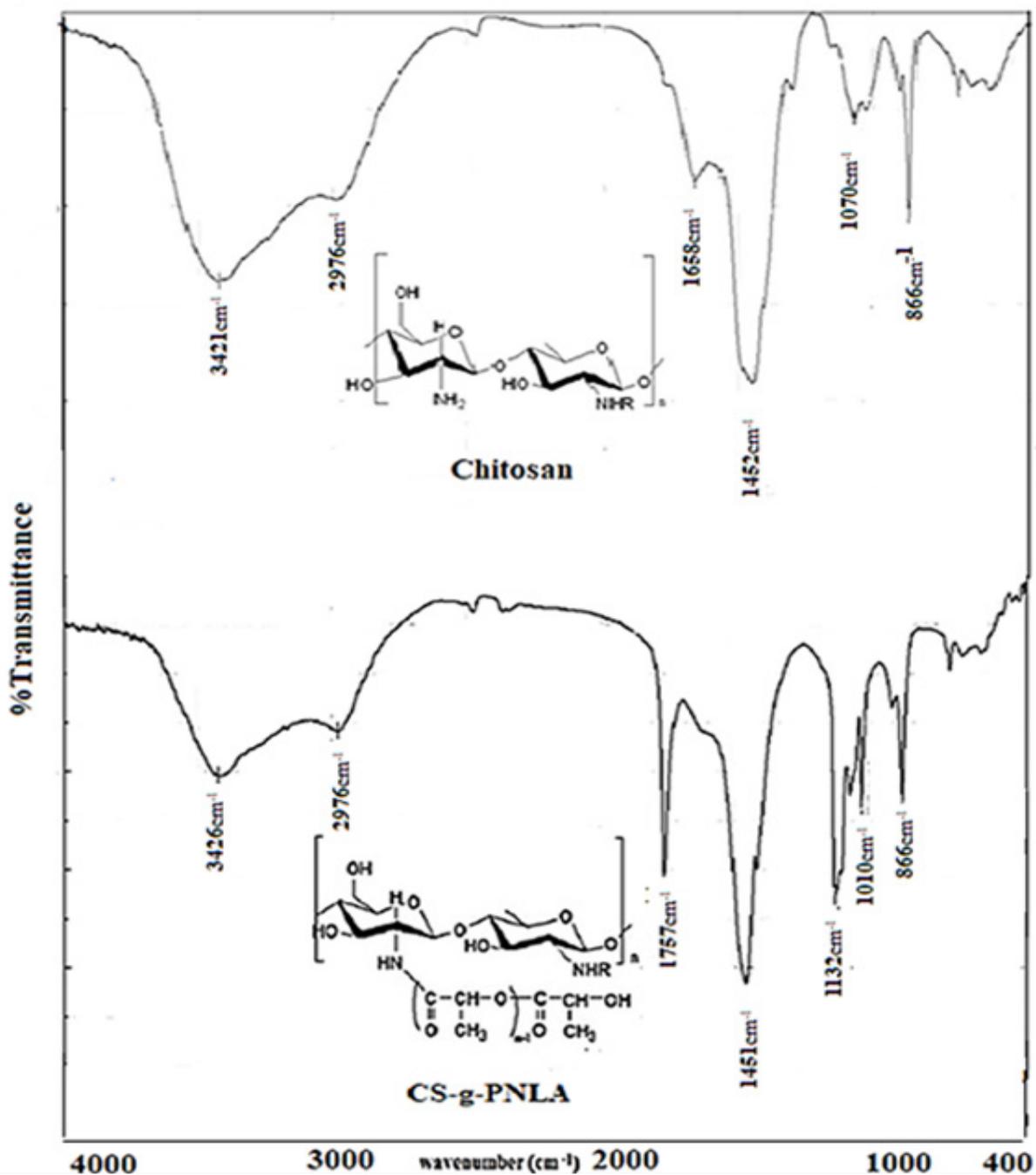


Figure 1. FTIR spectrum of chitosan and CS-g-PNLA copolymer

MOLECULAR RESULTS

Absorption spectra of DNA complexes with chitosan and CS-g-PNLA copolymer were employed to study the concentration changes associated with the

interaction between the polymer and the DNA. Results obtained are shown in Figure (2).

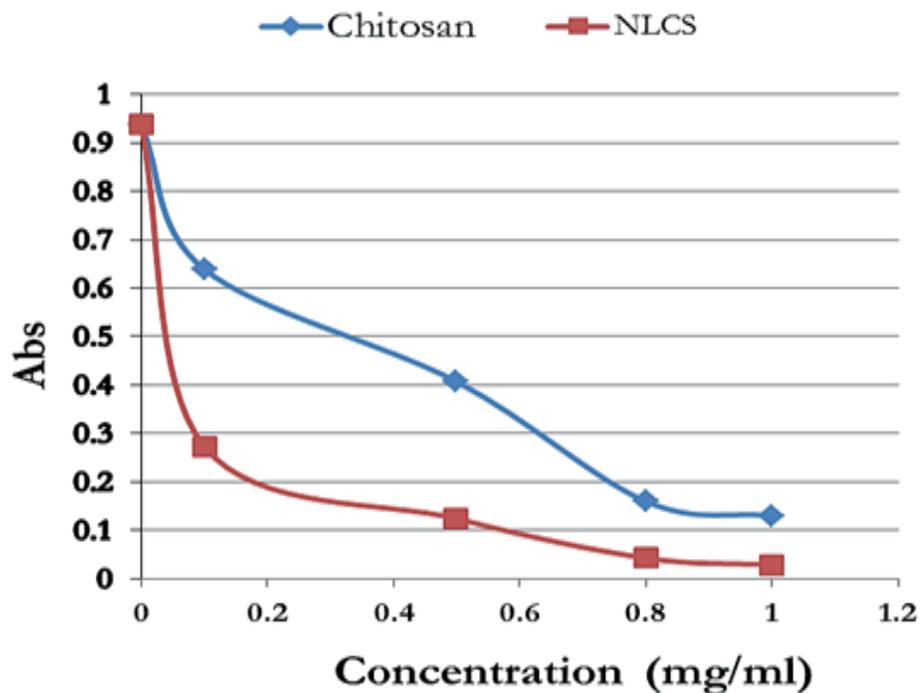
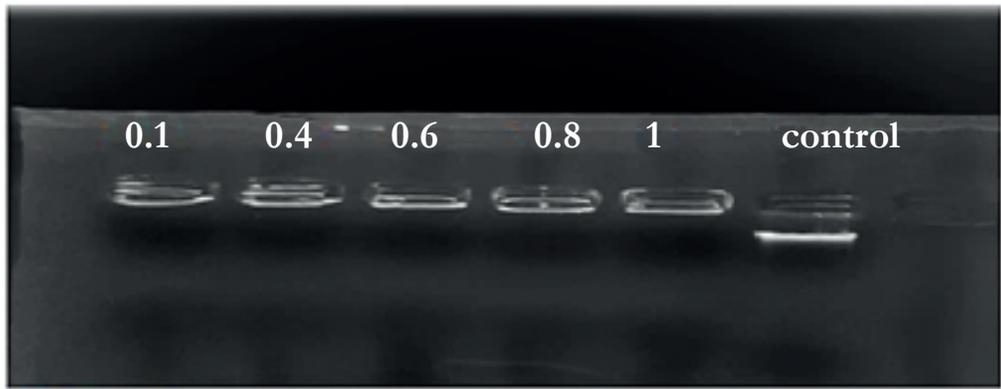


Figure 2. Effect of different concentration of chitosan and CS-g-PNLA copolymer on the DNA absorbance

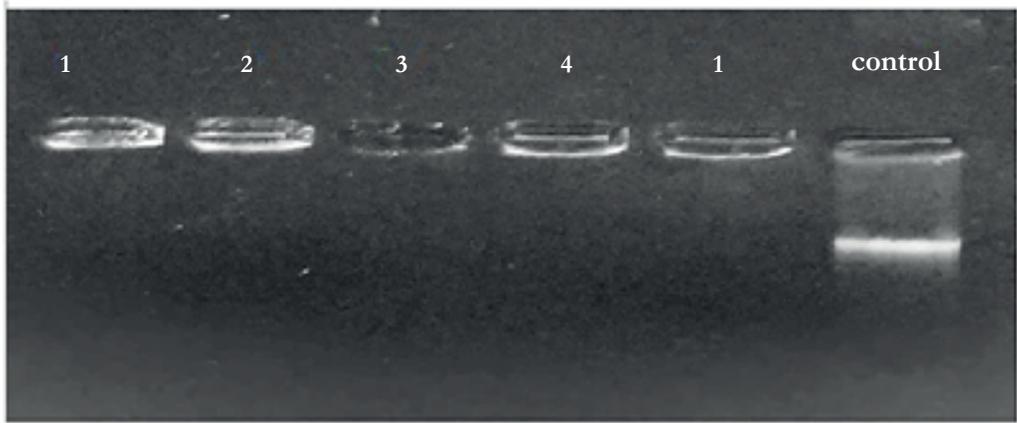
The binding affinity for serial concentrations of chitosan/DNA and CS-g-PNLA copolymer/DNA complexes with human DNA were shown in Figure (3).

THE BINDING ABILITY OF CS-G-POLY(N-LACTIDE) GRAFT COPOLYMER WITH HUMAN GENE

The heat shock protein gene (*hsp-70*) with 590 bp. in length⁽²⁰⁾ was used to study the binding ability of CS-g-PNLA copolymer with human genes. The effect of prepared polymer on gene was studied by gel electrophoresis as shown in Figure 4.



Chitosan



CS-g-PNLA

Figure 3. Gel electrophoresis of chitosan and CS-g-PNLA copolymer

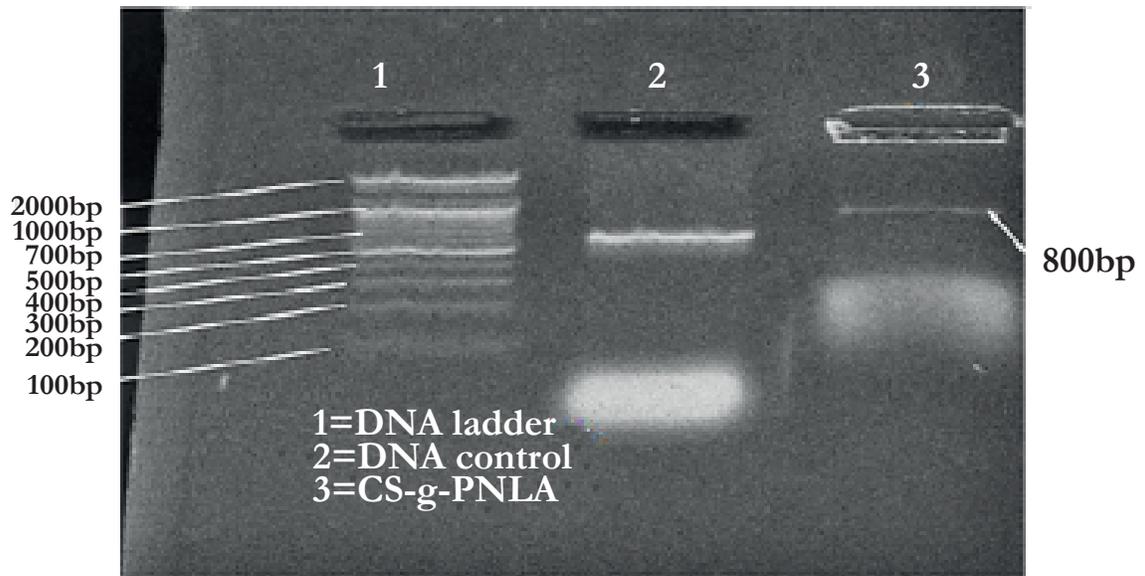


Figure 4. PCR product of hsp-70 gene complex with CS-g-PNLA copolymer

DISCUSSION

FTIR spectrum of chitosan shows a broad band between 3600-3000 cm^{-1} related to the stretching vibration of hydroxy and amino groups, and a weak band with a peak at 1658 cm^{-1} assigned for amide I which was left from deacetylation process. Figure 1 also shows absorption around 1070 cm^{-1} assigned to the C-O-C glycosidic linkage. The other two peaks at 2975 cm^{-1} and 1450 cm^{-1} represent stretching of the aliphatic C-H bond of methylene groups and bending aliphatic C-H bond for CH_2OH groups, respectively. While Figure 2, exhibits the FTIR spectrum of the prepared chitosan-g-poly(N-lactide) graft copolymer. In comparison with Figure 1, it indicates the appearance of a new absorption peak at 1758 cm^{-1} , assigned for stretching vibration of the carbonyl ester groups of the grafted poly (lactide) onto chitosan (24). This confirmed that the grafting process of L-lactide onto chitosan by ring opening polymerization took place.

Examining Figure 3, one can observe that the absorption of the DNA-polymer complex is decreasing with increasing the polymer concentration in comparison with the absorption of the DNA control. It was assumed that each phosphate group of DNA is a binding site and that each protonated group of polymer molecule can interact with more than one binding site on DNA (25,26). The results obtained, demonstrating that the binding density increases by increasing polymer concentration, and when the number of neutralized charges on DNA exceeds a critical number, the interaction can permit to apply it in DNA delivery system (non-viral carriers), especially with the presence of chitosan which is considered one of the most common natural polymers studied in non-viral gene delivery (27,28).

The migration of DNA in the gel was observed as the ratio of the polymer was increased, this observation demonstrating that the studied polymer is capable of binding to the DNA at a normal human temperature, i.e. 37 °C, and low pH value, although there was a migration of free DNA higher than the copolymers-DNA complexes.

Complexation between DNA and the protonated polymer has been characterized in terms of several features such as electrostatic interactions (29,30). Understanding the DNA-polymer interaction and the stability of these complexes in media that are relevant to their in vitro properties and in vivo application is essential for the optimal design of the synthetic vector (31,32).

The PCR product of hsp-70 gene was about 590 bp. in length. Meanwhile, the gene-polymer complexes were raised to about 800 bp. due to the interaction of these polymers with the gene that prompted us to develop non-viral means of delivering genes in vivo.

CONCLUSIONS

1. The prepared CS-g-PNLA copolymer has safety effects on human DNA and genes.
2. It shows high efficiency to electrostatic interaction with human DNA and gene.
3. This may make the copolymer be use as DNA and gene delivery.

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