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Preparation of some Chitosan Derivatives and Study Their Effect on Human Genetic Material

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ABSTRACT

Chitosan is a polycation biopolymer material. It has several applications, especially in the biopharmaceutical and biomedical fields. Chitosan was produced from shrimp waste by chemical method involving demineralization, deproteinization and deacetylation. It was characterized by intrinsic viscosity measurement and Fourier Transform Infra-Red in order to determine the average molecular weight and degree of deacetylation of purified Chitosan. Chitosan was modified by grafting process with maleic anhydride, poly(adipic anhydride) and poly(sebacic anhydride) to evaluate their effect on binding to the human genomic DNA. The grafted chitosan derivatives appeared as promising materials to be used as a model for DNA and gene delivery. Grafted chitosan-DNA complexes were determined by gel electrophoresis technique and ultraviolet spectroscopy.

INTRODUCTION

Polymers play an important and ubiquitous role in everyday life [1]. Polymers have different resources ranging from synthetic plastics such as polyethylene to natural polymers such as DNA and proteins that are essential to biological structure and function [2].

In the recent decades, polymers are widely used as biomaterials due to their favourable characteristics of an easy preparation, design and good biocompatibility. A variety of structures and interesting bio-mimetic character so the researchers develop a type of polymers called smart polymers [3].

Polymers can be specifically designed for the proposed application related to their structures and chain lengths (i.e. molecular weights), such for coupling of cell- or tissue-specific targeting moieties or performing other alternations that grant upon them a specific physiological or physicochemical properties.

Cationic macromolecules used for nucleic acid delivery acquire their charge, which are capable of forming electrostatic complexes with DNA under physiologic circumstances. For example, poly(L-lysine) (PLL) and its derivatives [4,5] contain primary amines; polyamidoamine (PAMAM) starburst dendrimers [6,7] have primary and tertiary amines; branched polyethylene-imines (PEI) have different amino groups, while linear PEI have mostly secondary amines [8,9]. Diethylaminoethyl (DEAE) dextran possess tertiary amines [8]; chitosan and its derivatives have primary amine or modified quaternary groups [9,10]. Some of the most studied polycations used for gene delivery are [11].

Smart polymers respond to common stimuli of three types: physical, chemical or biological [12]. Temperature and pH increase in ionic strength, presence of specific metabolic chemicals, the addition of an oppositely charged polymer and polycation-polyanion complex structure, changes in electric [13] and magnetic field [14], light [15] or radiation forces. Stimuli responsive macromolecular materials have attracted great interest due to their clear applications in biomedicine and biotechnology [16].

In this work, chitosan was extracted from chitin of the shrimp shells wastes, and it was modified by grafting process in toluene by maleic anhydride, poly(adipic anhydride) and poly(sebacic anhydride). The influences of the chitosan derivatives to bind to the human genomic DNA were evaluated by gel electrophoresis technique and ultraviolet spectroscopy.

MATERIALS AND METHODS

Extraction of Chitin

One hundred grams of shrimp shells were dried at 100°C for 10 hours in a drying oven, and then ground to powder, Then 1000ml of (5%) hydrochloric acid solution was added the powder and stirred mechanically for 24 hours at room temperature to remove minerals and their salts. The residual powder was filtered and washed several times with distilled water to remove residual hydrochloric acid. Then, 1000ml of (10%) sodium hydroxide solution was added to the powder and stirred mechanically for 3 hours at 90°C to remove all protein. The mixture was cooled down at room temperature. Filtered and washed several times with distilled water to remove residual sodium hydroxide and dried to obtain chitin powders [17].

Deacetylation of chitin to chitosan

Two procedures were used to convert chitin to chitosan aiming to get better degree of deacetylation and then good solubility of chitosan. Firstly, 20g of chitin was mixed with 250ml of (50%) potassium hydroxide solution and charged in three-neck round bottom flask fitted with a condenser, mechanical stirrer and thermometer. The mixture was heated to 90°C for 6 hours. Finally, it was filtered, washed with water and dried in an oven at 45°C [17].

The second procedure involved mixing of 20g chitin and 250ml of (50%) sodium hydroxide solution and then left to boil at 110°C for 3 hrs. Then, it was left to cool down for 30 minutes at room temperature. Afterwards the sample is washed continuously with 50% NaOH and filtered in order to retain the chitosan [18].

Purification of chitosan

Two grams of chitosan dissolved in 1% acetic acid and stirred until a homogenous solution is obtained and the insoluble were removed by filtration. Chitosan 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 [18].

Preparation of Chitosan derivatives

N-maleoyl chitosan (NMCS), N-adipoyl chitosan (NACS) and N-sebacoyl chitosan (NSCS) were prepared by grafting process. It involves dissolving 1g from each, maleic anhydride, poly(adipic anhydride) or poly(sebacic anhydride) with 100ml of freshly distilled toluene added to 1g of chitosan. The mixture was stirred and refluxed for 2 hours, filtered and washed with acetone for several times then dried in the vacuum desiccator [19,20]. The white powder products of (NMCS), (NACS) and (NSCS) were obtained with %yield about 68.9, 60.3,and 51.1 respectively. They were characterized by FTIR spectroscopy.

Molecular Study

Samples collection

Three ml of venous blood was collected by vein puncture in EDTA tube from voluntary peoples, and then used freshly to extract the human genomic DNA.

DNA extraction

Human genomic DNA was extracted from all fresh blood samples using the DNA extraction Mini Kit (Geneaid Company), according to the Kit protocol. DNA quantity and quality were assessed by Nanodrop (Nanodrop/optizen system), at 260/280 nm respectively.

DNA samples then visualized by 0.8 % agarose gel electrophoresis according to[21]using the reagents: 1X-TBE buffer, which stained with ethidium bromide

The effect of prepared polymers on human genomic DNA

The effect of the studied polymers on DNA was performed by mixing 10 µl of DNA with 10 µl of polymer solutions in different concentrations of each polymer which are:(0.1,0.4,0.6,0.8 and 1) mg/ml (2% HAC), the mixture then incubated at 37°C for 1 hour. The DNA concentration per nucleotide was determined by UV absorption spectroscopy at 260 nm. to be calculated the percentage of binding nucleotide in DNA samples with polymers.

The polymer – DNA mixture were electrophoresed for 1 h at 60 Von a 0.8 % agarose gel in 1X TBE buffer. The gel was stained with ethidium bromide and photographed under UV light.

RESULTS AND DISCUSSION

Degree of deacetylation (DD)

Degree of deacetylation is one of the main parameters characterizes chitosan, and it is related directly to the quality and solubility of chitosan, then higher solubility will produce a better chitosan [22].

There are different methods for measuring the degree of deacetylation of chitosan [23] and several equations are described for calculation of the degree of deacetylation. The simplest method is measured by absorbance baseline method using infrared spectroscopy [4].In this procedure, calculation of DD depends on the absorbance ratio band at 1655 cm^{-1}due to amide and at 3450 cm^{-1}refers to amine absorbance using equations (a) [24] and (b) [25]:

$$DA = \frac{A1655}{A3450} \times 100 \,/\, 1.33 \qquad \text{.....(a)}$$

Then, DD = 100 – DA or it may estimate directly from the following equation:

$$DD = \frac{1.33 - A1655/A3450}{1.33} \times 100 \qquad \text{.....(b)}$$

High degree of deacetylations was obtained especially with purified chitosan. The results obtained for DD and DA of chitosan and chitin are listed in Table (2).

Infrared spectra of chitin and Chitosan

The FTIR spectrum of chitin is exhibited in Figure (1). It shows a clear, broad band with a peak at 3445 cm^{-1} which assigned for hydroxyl groups stretching and a peak at 2926 cm^{-1} assigned for aliphatic C-H bond stretching of methylene group's, and at 1380 cm^{-1} due to amide C-H bending. The stretching of carbonyl amide I group appears as a sharp peak at 1660 cm^{-1} and a peak at 1562 cm^{-1} related to N-acetyl ester bond of amide II and at 1070 cm^{-1}indicates C-O-stretching [26,27].

FTIR spectrum of pure chitosan is also shown in Figure (1). It exhibits a broad peak at 3422 cm^{-1} due to -NH stretching and -OH stretching, a weak band peak at 1658 cm^{-1} assigned for amide I which was left from deacetylation process. No peak or very weak is shown in the region of 1560 cm^{-1} which was assigned for N-acetyl ester in chitin spectrum, this is confirm that chitosan with high degree of deacetylation was obtained. Also, the appearance of the new band at 1452 cm^{-1} peak due to C-N stretching bond confirms the deacetylation process.

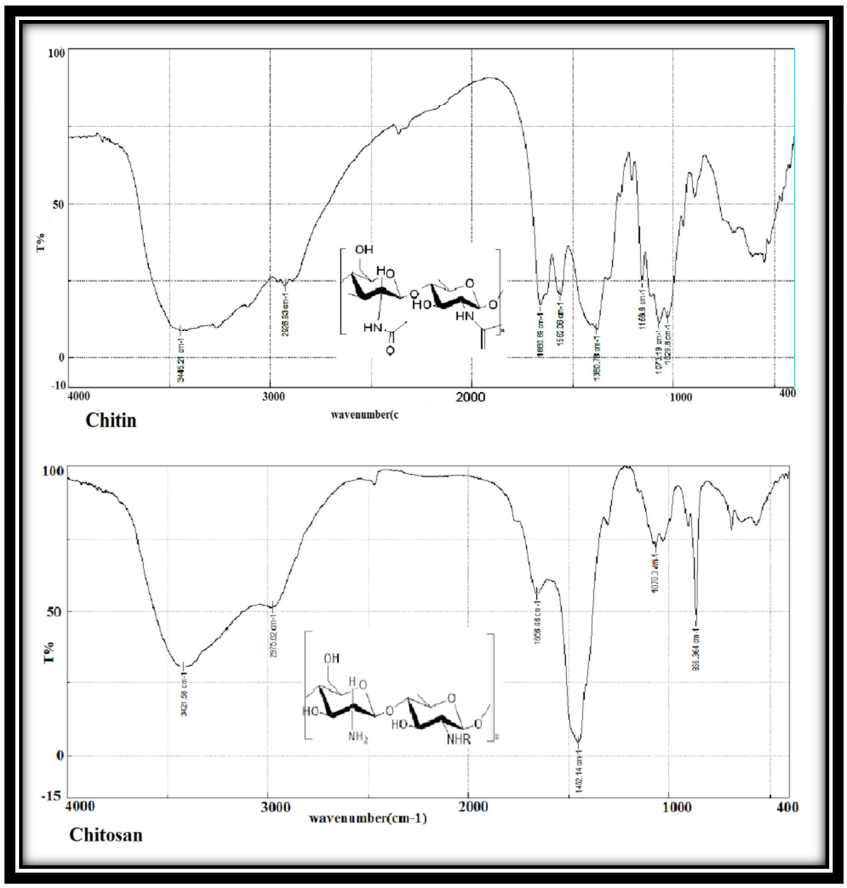

Figure (3.1): FTIR spectra of chitin and chitosan

In this study, two sets of chitosan samples deacetylate by different media and heating conditions, namely 50% KOH and 50% NaOH. Results obtained demonstrate which of those procedures yield reliable results, and also to assess their advantages and disadvantages by direct solubility behavior comparison the values of DA and DD. The target was to obtain soluble chitosan and derivatives to be used for different bio applications. This was verified in obtaining soluble chitosan with higher DD above 90%, Table (2). The values obtained using these proposed relationships agree closely with those reported in the literature [24, 25, and 28].

Table (2): Degree of deacetylation and degree acylation of chitin and chitosan according to equations a & b

Samples	DA (a)[1]	DD (a)[1]	DA (b)[2]	DD (b)[2]
Chitin	61.89	38.11	61.66	38.34
Chitosan (KOH)[3](Slightly soluble)	52.86	47.14	52.86	47.14
Chitosan (NaOH)[3](Moderately soluble)	21	79	21.1	78.9
Chitosan (NaOH)[3] (Highly soluble)	9.02	90.98	9.03	90.97

[1]Calculated from equation (a), and [2]Calculated from equation (b)
[3]The alkali solution used for deacetylation

3.1.3 Molecular weight of Chitosan

The molecular weight of chitosan is measured by viscosity method using an Ubbelohde Viscometer. It is known that the intrinsic viscosity [η] as a function of viscosity average molecular weight M_v is represented by Mark-Houwink-Sakurada equations [29]:

$$[\eta] = k M_v^{\alpha}$$

Where k and α are constants and their values depend on the type of polymer, solvent and temperature. Under working conditions, $k = 9.66 \times 10^{-5}$ dm^3g^{-1} and $\alpha = 0.742$ [30].

Figures (2)show the linear plot of calculating reduced viscosity versus concentration for chitosan in the solubilizing aqueous mixture of ammonium acetate/acetic acid. The slope is representing the intrinsic viscosity and was found to be equal to 2.178. The calculated chitosan viscosity average molecular weight from Mark-Houwink-Sakurada equation is equal to $7.4x10^5$ g/mole. However, this value is considered as a moderate value comparing to the lower value of $3.9x10^5$ obtained by Yacob et al [30], and the higher value of $10.5x10^5$obtained by Hossain and Iqbal [22]. This is measured, of course, for the chitosan sample having the highest degree of deacetylation shown in Table (2) due to its higher solubility in the acidic buffer solution.

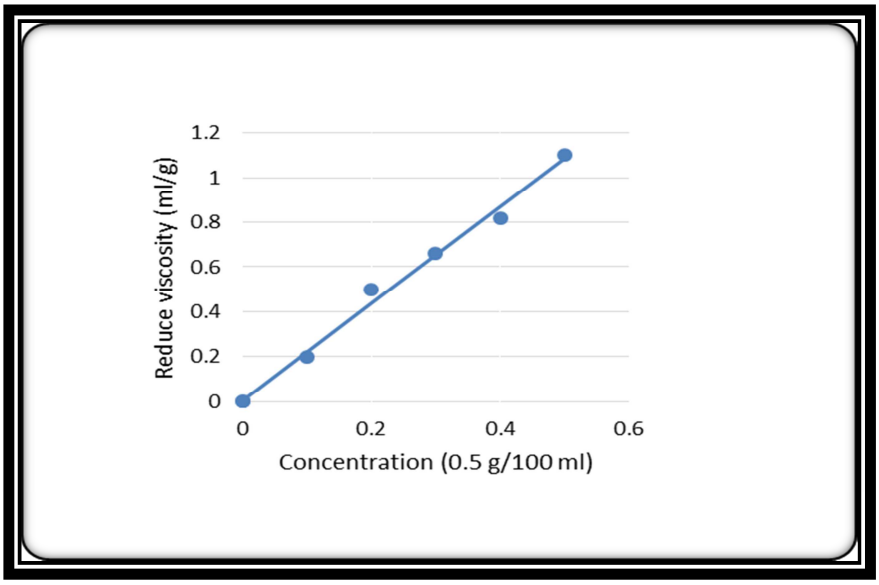

Figure (2): Linear relationship between reduce viscosity and different aqueous ammonium acetate/acetic acid solutions of chitosan at 25°C

Characterization of Chitosan Derivative

Grafting of chitosan allows the formation of functional derivatives by covalent binding of a molecule, the graft, on to the chitosan backbone. Chitosan has an amino reactive group that can be grafted [31,32], and this is the case here. The added anhydrides, maleic, adipoyl and sebacoyl expected to interact with the amino groups to form the following derivatives, N-maleoyl chitosan (NMCS), N-adipoyl chitosan (NACS) and N-sebacoyl chitosan (NSCS) respectively.

Examining the FTIR spectrum of N-maleoyl chitosan (NMCS), Figure (3), one can easily see that a new band appears in the region of 1715 cm^{-1} assigned for carboxylic acid carbonyl groups which confirm the ring opening of maleic anhydride, and a peak at 1619 cm^{-1} assigned for stretching C=C double bond of maleic anhydride, and a peak at 1323 cm^{-1} due to C-O of carboxylic acid comparing to the FTIR of un-grafted chitosan only beside the other fundamentals band [26,27], Figure (1).

Grafting chitosan with poly(adipic anhydride) and poly(sebacic anhydride) produces an interesting FTIR spectrum, especially in the region of aliphatic C-H stretching, Figure (3). Their FTIR spectra show a sharp peak with increasing intensity at 2929 cm^{-1} and 2928 cm^{-1} due to stretching of the methylene group bond of NACS and NSCS respectively, and peak at 1325 cm^{-1} for NACS, and at 1330 cm^{-1} for NSCS due to C-O of carboxylic acid groups. The most important bands starching and their assignment is also tabulated in Table (3).

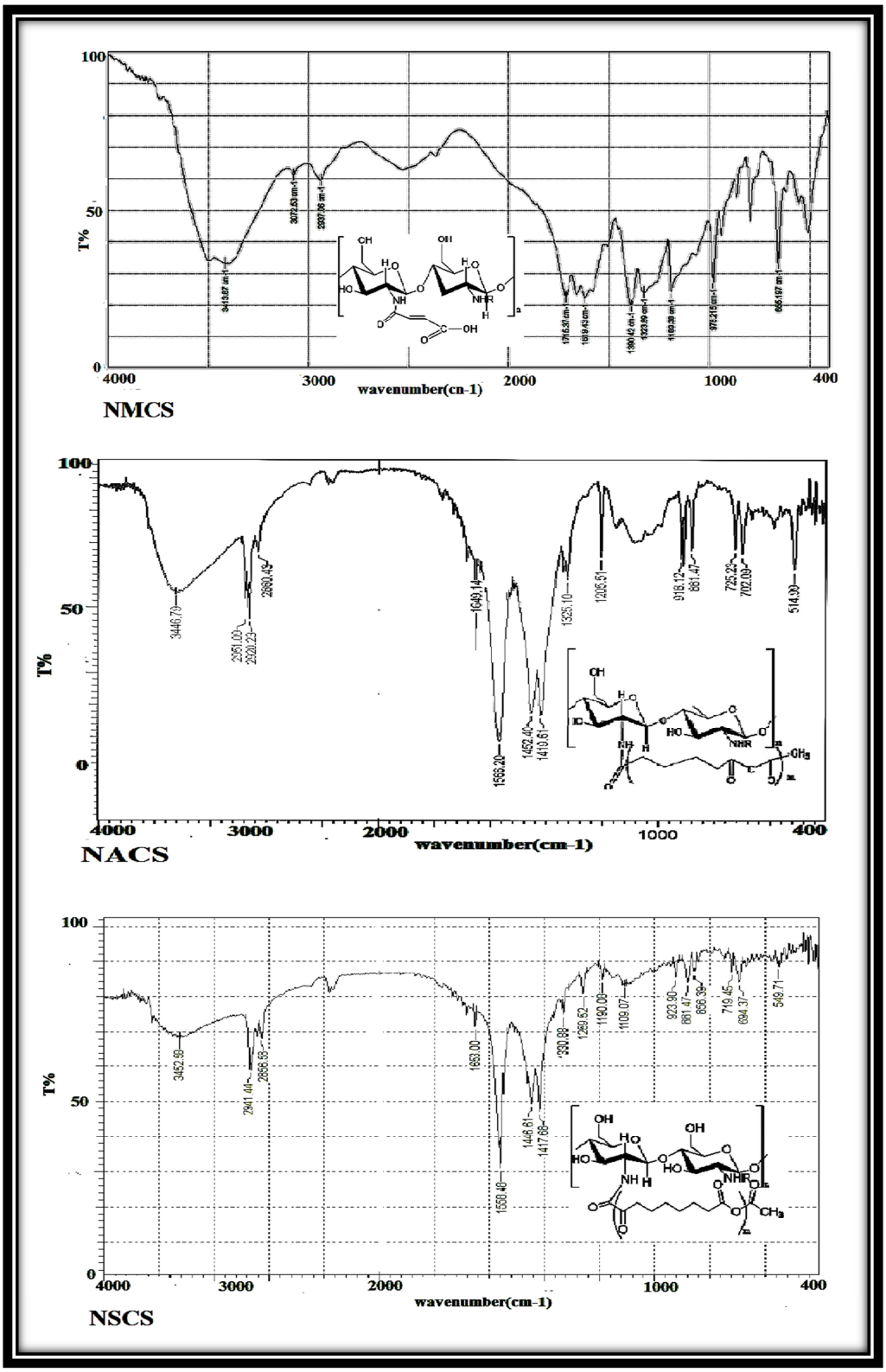

Figure (3): FTIR Spectrum of NMCS, NACS and NSCS

Table (3): The major IR bands to chitosan derivatives

Chitosan derivative	Functional Group	Assigned Bond	Stretching Frequency (cm^{-1})
NMCS	-CO-OH	O-H	3474
	-CO-OH	C=O	1715
	CH_2CH_2	C=C	1619
NACS	-CO-OH	O-H	3440
	CH_2	C-H	2928
	CO-O-CO	C-O	1330
NSCS	-CO-OH	O-H	3428
	CH_2	C-H	2929
	CO-O-CO	C-O	1325

Molecular results:

Human genomic DNA from many samples was extracted and its quantity and quality were summarized in Table (4).

Table (4): The quality and quantity of extracted genomic human DNA

Sample	A_{260}	A_{280}	Concentration	Ratio
1	0.265	0.157	13.250	1.688
2	0.160	0.095	8.000	1.684
3	0.129	0.081	6.450	1.613
4	0.223	0.136	11.150	1.640
5	0.212	0.125	10.600	1.696
6	0.096	0.051	4.800	1.882

Figure (4) shows the 0.8% agarose gel electrophoresis of the extracted DNA, the bands of total genomic DNA was observed as purified compact bands under UV light.

DNA

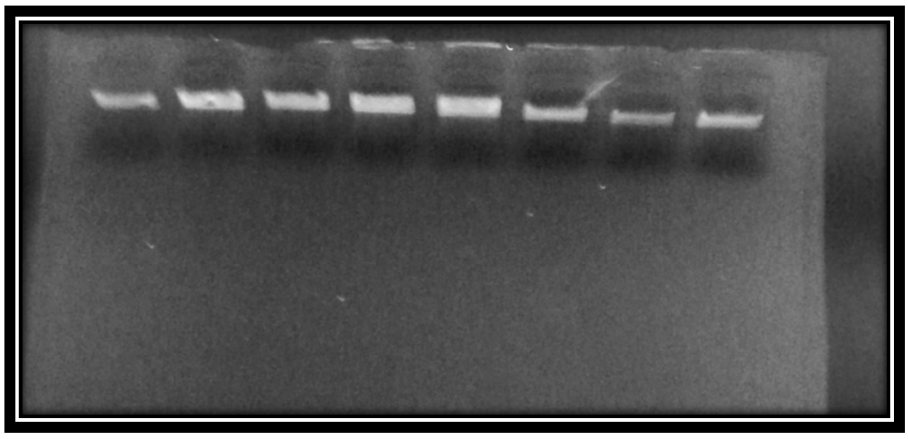

Figure (4): Total genomic DNA on agarose gel electrophoresis

The effect of chitosan and its derivatives were shown in figure 5, which illustrate the absorption spectra of DNA complexes with each of chitosan and its derivatives, the spectra shown the decreasing the absorption of nucleotides in DNA-polymer complex, with the increasing the polymer concentration in comparison with the absorption of the naked DNA, the results revealed that the chitosan and the derivatives have the binding ability with the genomic DNA and interaction with it. The binding mode may be due to the electrostatic interaction between the protonated amine group in each polymer and the negative charge phosphate group in the structure of the double strand DNA, several scientists studied the electrostatic binding between protonated polymers and DNA molecules [33-35].

On the other hand, the absorption percentage (% A) at 260 nm of the binding DNA with the polymers was calculated depending on the equation c:

$$\% A_B = \frac{A_{DNA} - A_{DNA\text{-}P}}{A_{DNA}} X100 \quad \text{……. (c)}$$

Where:
A_B= absorption of the binding nucleotide with the polymer
A_{DNA}= absorption of whole DNA without polymer
A_{DNA-P} = absorption of DNA-polymer complex

Figure (5), showed the capacity binding or conjugated of the polymers in different concentrations with the genomic genetic material (DNA), the spectra appear that different concentration in each polymer has the high binding behavior with the DNA.

The results of our study enhanced us to expect that these polymers can be choice with an appropriate concentration, as DNA delivery system (non- viral carriers), especially with the presence of chitosan which consider one of the most commonly studied polymers in non-viral gene delivery [36].

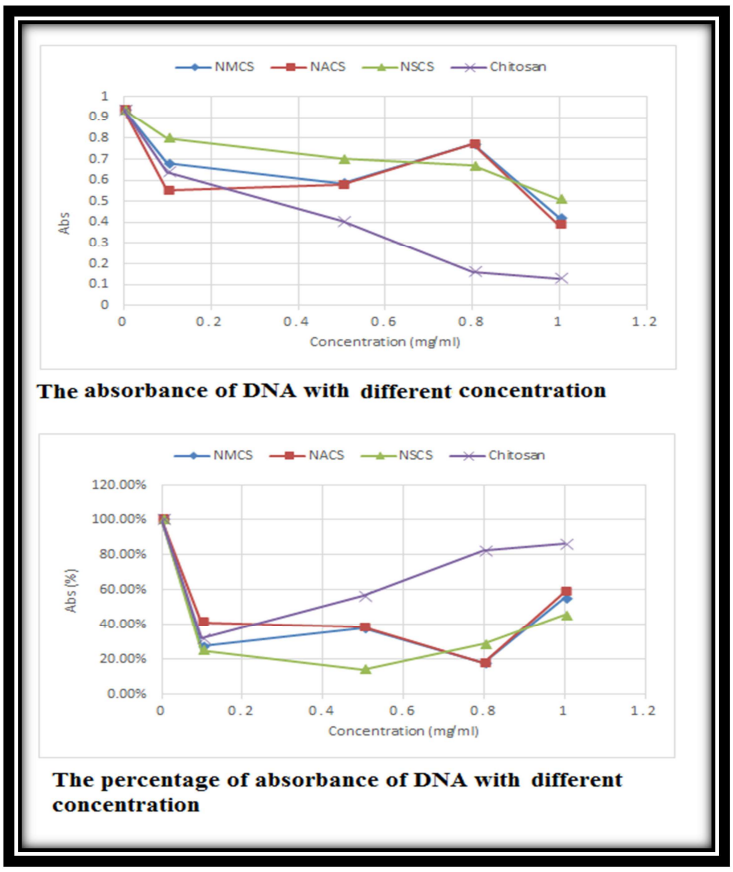

Figure (5): Absorbance of chitosan and it's derivatives in different concentration

Gelelectrophoresis technique in different concentrations of each polymer, has been performed on a DNA, DNA-chitosan, DNA-NMCS, DNA-NACS and DNA-NSCS, respectively, Figure(6), the migration of DNA on agarose gel were done under the influences of electrical field by which the DNA molecule moved faster than the DNA- polymer complexes, that appear a retarded band when the binding with double strand DNA. Many scientific researches discuss the retards behavior of the DNA migration in agarose gel electrophoresis and they suggest that the binding between DNA and polymers is thought to occur mainly through electrostatic interactions among the participating polymers [37-39].

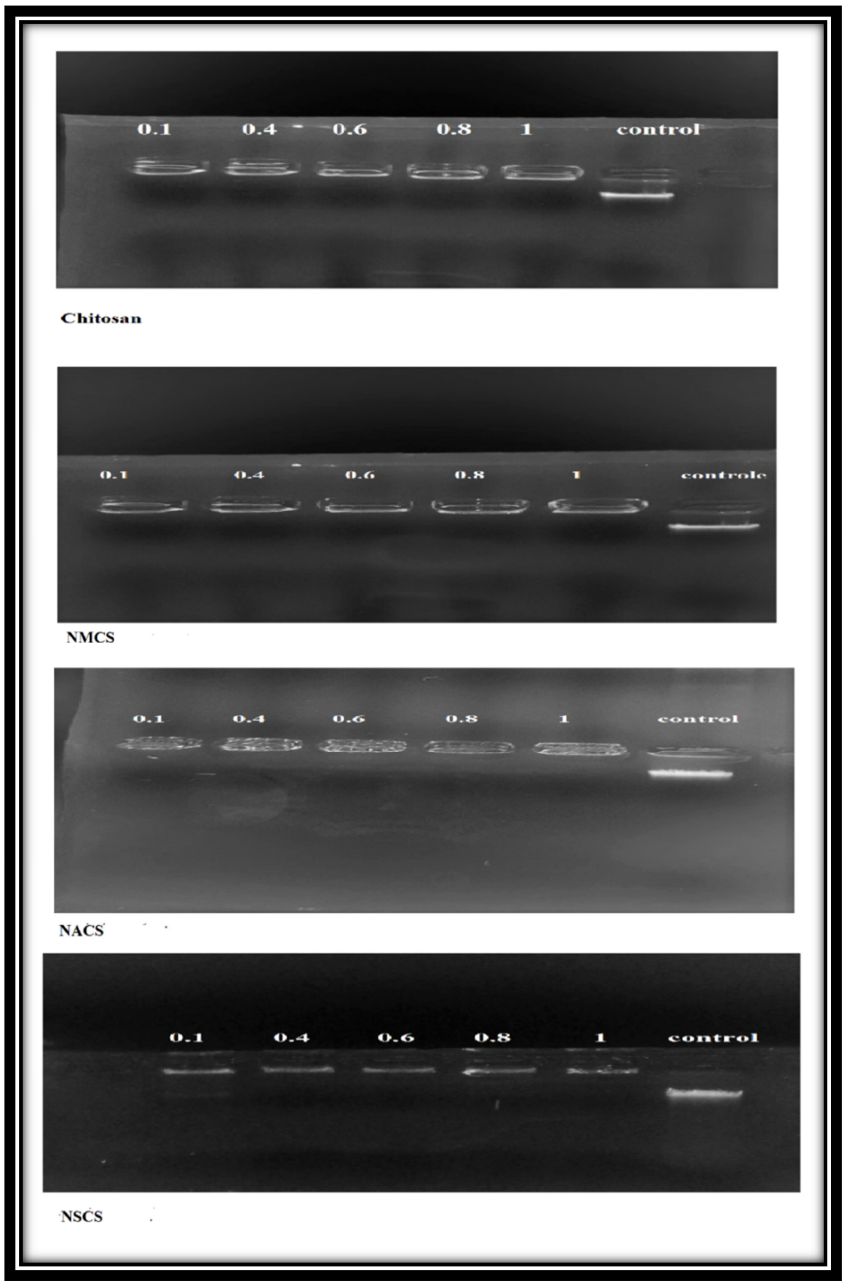

Figure (6): Gel electrophoresis of chitosan and its derivative

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