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Research Article

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Cellular uptake of chitosan nanospheres by HEP G2 cells phagocytosis

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ABSTRACT

No reports have discussed this point before that. Chitosan nanospheres (CNPs) with an average size of 110 nm and positive potential of +27.8 mv have been prepared via chemical ion gelation method and characterized by spectroscopy and electron microscopy. Hep G2 cell line is a suitable host for Hepatitis C Virus (HCV) replication. Hep G2 was treated with a concentration of $100\mu g/mL$ (w/v) of CNPsto investigate their effect on Hep G2. Visible cell aggregations and granulations took place in the tissue culture flask after 48 hours. This may be mediated, in part; by Hep G2 phagocytosis, which has been recorded with electron microscopy 24 hours post treatment. Thus, CNPs might attain the critical aim of drug delivery in a persistent HCV culture.

Key words: Chitosan, Nanospheres, Hep G2, Phagocytosis, anti-HCV Drug Delivery

INTRODUCTION

Nano-biology is a newly emerging, promising and vastly expanding area of science. This area of research is developing novel applications which can work efficiently as drug carriers, aid in reporting the outcome of therapy and provide real-time evaluation of therapeutic efficacy. The main objective of this work was the using of chitosan nano-particles (CNPs) as a smart drug delivery system for antiviral HCV drugs, using hepatoma cell line (Hep G2) as a replicative host for the virus, in order to enhance their activity and decrease their side-effects. However, during the study Hep G2 cells were illustrated to uptake chitosan nanospheres by phagocytosis using transmission electron microscope.

Chitosan Nanospheres

Chitosan and its nanoparticles attract a marvelous attention as a potentially important, ubiquitous, inexpensive, renewable resource, biodegradable, biocompatible and have been extensively applied in the biomedical, pharmaceuticals, agricultural, environmental protection and biotechnology(1,2,3,4,5).

Chitosan is the most important derivative of chitin. Chitin is the main constituent, 70%, of the arthropods exoskeletons, and fungi cell walls. Chitosan was usually extracted from marine crustacean exoskeleton by partial deacetylation in the solid state under alkaline conditions, or by enzymatic hydrolysis(3, 6).

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The term chitosan does not refer to a distinctively defined compound; it simply a family of copolymers. Chitosan is a high molecular weight linear cationic hetero-polysaccharide consisting of linear chain of two monosaccharaides, N-acetyl-D- glucosamine and D- glucosamine, linked by β -(1 \rightarrow 4) glycosidic bonds. The heterogeneity of chitosans may be attributed to the variable ratio of the two monosaccharaides in the chitosan linear chain, leading to many differences such as degrees of deacetylation (DD), 75-95%, molecular weights (MWt.), 50-2000 KDa, viscosities, etc.(7).

Hep G2 cell lines

Hep G2 (ATCC HB 8065) is a human (*Homo sapiens*) hepatoma derived cell line. The cells were obtained from liver epithelial cells of 15 years adolescent Caucasian male with a hepatocellular carcinoma (HCC). Hep G2 is not tumorigenic, and suitable as a transfection host (**8**).

EXPERIMENTAL SECTION

Synthesis of CNPs

Chitosan and CNPs have been prepared according to (9)by ion gelation method (10) using Tri poly phosphates (TPP) as the reducing agent in an aqueous medium. TPP initially acts as a reducing agent then acts as the stabilizing agent by forming a layer or cap over the CNPs surface, inducing enough electrostatic repulsion between individual particles to keep them well dispersed in the medium and prevents aggregation or further growth of the particles.

Characterization of CNPs

The study of the features of synthesized molecules was accomplished by High-Resolution Transmission Electron Microscope (HR-TEM) Joel JEM-2100 microscope (accelerating voltage 200kV; Gatan Erlangshen ES500digital camera) to define the ultra-fine structure, particle size, shapes and size distribution profiles of CNPs. To determine the charges the Zeta–sizer, Nano Series, Nano ZS, Malvern Co., UK was used.

Hep G2 Cell line

Hep G2 cells were purchased from The Holding Company for Biological Products & Vaccines (VACSERA), Giza, Egypt. The American Type Culture Collection (ATCC) number is HB8065. Cells obtained from VACSERA were a monolayer sheet in 20 mL of RPMI growth media supplemented with 10 % FBS The cells were routinely propagated and maintained, as well as cells viability and plates preparation were carried out in the tissue culture laboratory, at National Cancer Institute (NCI), Cairo University, Cairo, Egypt.Hep G2 cells were examined under inverted light microscope (Eclipse Ti, Nikon Co., Japan) to compare cell appearance and morphology before and after treatment with CNPs through time intervals.JEOL Transmission electron microscope(JEM-1230, Japan, Full computerized, with more than 600 KX magnification power and 0.5 nm resolving power) has been used for electron microscopy studies on the Hep G2 cells.

RESULTS

Morphology of CNPs by HR-TEM

Size or diameter and shape of CNPs capped with TPP have been measured and determined using HR-TEM imaging, as soon as possible after the preparation by ion gelation chemical method.CNPs (**Figure 1**) appeared to be amorphous or slightly spherical in shape, and looked rough. Most of chitosan nano spheres were dispersed or evenly distributed in the microscopic field, however some particles were aggregated.The histogram chart (**Figure 2**) provided the size distribution of synthesized CNPs was plotted by recording the sizes of a great number of particles on the TEM images; it showed that the size distribution of nanoparticles ranged from 50 nm to 130 nm. The average most frequent size of the prepared CNPs was about 110 nm.

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Figure 1: HR-TEM image of CNPs capped with TPP. Right (scale bar 200 nm): particles show slightly rough spherical or amorphous shapes. Left (scale bar 500 nm): .particles were dispersed or aggregated



Figure 2: Histogram which shows the average particle size 100-120 nm

Charges and sizes of CNPs by Zeta-sizer:

CNPs charge or potential was measured by Zeta-sizer. As shown in the Zeta potential distribution report (Figure 3)CNPs were cationic and positivity charged by a value of $+27.8 \pm 6.76$ mv.



Figure 3: Zeta-sizer report of Zeta potential distribution. The potentials of CNPs were presented in one peak with a positive value of $+27.8 \pm 6.76$ mv

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Effect of CNPs on Hep G2 morphology

Certain changes in morphology and appearance of Hep G2 cells (**Figure 4**) post inoculation with CNPs in compare to moc-treated, untreated, cells (MHep G2) were observed by microscopic examination under inverted microscope. Such visible morphological changes were the granulation and cell aggregation that took place especially after 48 hours incubation period.



Figure 4: Hep G2 Cell morphology post inoculation with CNPs. Right: after incubation period of 48 hours. Left: MHep G2 cell line after 48 hours of incubation

In vitro cellular uptake detected by TEM

The ability of the particles to enter into the cells and be reserved within the cell is important to achieve the aim of drug delivery. After the examination of transmission electron microscope (TEM) images (Figure 5), the established binding, and internalization of CNPs in Hep G2 cells have been illustrated as following:

Chitosan nano spheres have been detected in more than one site may be separated or aggregated in clusters. Most of CNPs were usually agglomerated in clusters on plasma membranes, such as cell membrane and nuclear envelope. CNPs clusters were stick on the cell plasma membrane and this has been demonstrated in (Figure 5 A, B). Some CNPs were separated inside cytoplasm or outside the cell (Figure 5 A).

Hep G2 cell was engulfing the chitosan nano spheres besides the cell membrane (**Figure 5** C). Such a beautiful cellular engulfment firmly confirmed Validity of Nano-Chitosan as a Drug Carrier versus Hepatitis C Virus Replication *in vitro*.



Figure 5: TEM images of CNPs treated Hep G2 cell incubated for 24 hours. A: scale bar 2 μm. B: scale bar 0.5 μm. C: scale bar 178nm. Blank Arrows: CNPs outside the cell. Arrows 1: CNPs stick to CM (Cell Membrane). Arrows 2: Engulfment of the CNPs by Hep G2 cells. Arrow 3: CNPs Phagosome inside Cyto (Cytoplasm). Arrows 4: CNPs stick to NM (Nuclear Membrane). Arrows 5: CNPs were clustered on NM. Arrows 6: CNPS aggregations on CM. N: Nucleus

DISCUSSION

CNPs have been prepared via chemical ion gelation method and were characterized using HR-TEM and Zeta-sizer. The nanoparticles TEM image indicates that the particles were amorphous or slightly rough spherical in shape with average size of about 110 nm in diameter. By observing the Zeta-sizer report of CNPs, the particle diameter was presented in one main peak. This indicated the homogeneity of the prepared particles and absence of contamination with other principal sizes.

CNPs charge or potential by Zeta-sizer showed its positivity by a value around $+27.8 \pm 6.76$ mv. The CNPs positive potential value may be attributed to the amino groups present in chitosan. A positive charge of chitosan and nano chitosan is a well-known physical property of such a cationic compound and has been reported as variable in literatures.

Sizes and charges similar to our results have been prepared (11), who studied the potent therapeutic effect of chitosan nanoformulations on human immunodeficiency virus (HIV) but the particles were smooth, may be due to high quality of commercial chitosan used, slightly larger in size 130 nm, and charge + 30 mv. A positive potential of + 52 mv has been administrated according to (12) who has reported the same rough and round dense shapes of CNPs with a diameter of 65 nm. However, (10) prepared CNPs were ranging from about 313–412 nm with a positive potential range of 20–28 mv.

CNPs treated Hep G2 cells change in morphological appearances such as visible cell aggregation and granulation that took place 48 hours post inoculation with a $100\mu g/mL$ (w/v) concentration of CNPs that may be due to phagocytosis after successful treatment. Same morphological appearance of Hep G2 cells had been reported by (13) after 21 days of incubation of HCV-infection cells and was attributed to successful infection.

Cellular uptake of Nano-Chitosan by Hep G2phagocytosis

The ability of the particles to enter into the cells and be reserved within the cell is important to achieve the aim of drug delivery. After the examination of transmission electron microscope (TEM) images, the established binding, and internalization of CNPs in Hep G2 cells have been recorded.

Chitosan nano spheres have been detected separated or aggregated in clusters in more than site. CNPs were usually agglomerated in clusters on plasma membranes, such as cell membrane and nuclear envelope. Some CNPs were separated inside cytoplasm or outside the cell.

As shown in the TEM image; most of the nano-particles were agglomerated to each other and adherent to the cell plasma membranes, wither cell membrane or nucleus membrane. This superior adherence to cell membranes may be attributed to the electrostatic attraction forces between cationic CNPs and the negatively charged glycoproteins on the cell membranes. Visible cell aggregation and granulation that took place 48 hours post CNPs (100µg/mL (w/v)) treatment may be mediated, in part, by cellular phagocytosis.

Chitosan nano spheres have been engulfed by Hep G2 cell. Such beautiful cellular phagocytosis might illustrate the ability of CNPs to work as Trojan horse and carry the drugs for the cells.

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