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International Journal of Pharmaceutical Research & Allied Sciences, 2018, 7(3):189-197

Research Article

ISSN : 2277-3657
CODEN(USA) : IJPRPM

Evaluation of the Antioxidant and Anticancer Effects of Biodegradable/Biocompatible Chitosan–Alginate Nanoparticles Loaded with Vitamin C

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ABSTRACT

A safe, rapid, and easy-to-scale-up method of loading vitamin C (vit C) into chitosan–alginate nanoparticles (CH-Alg NPs) was established. The CH-Alg/vit C NPs were subjected to bath sonication to achieve their nanosize. Transmission electron microscopy of CH-Alg/vit C NPs stained with 1% neutral phosphotungstic acid showed NPs with sizes between 25–30 nm with spherical shapes. High negative zeta potential values (between -34 and -43) were obtained with a zeta analyzer. The main ingredients of the NPs were found to be chitosan and alginate. Various vit C concentrations were incorporated into the NPs to enhance the anticancer property of vit C. The effect of various pH values on the CH-Alg/vit C NPs was also studied to evaluate their degradation in the stomach and intestines. The results showed that pH ≈ 6 was the optimum level for polyionic interaction. The activity of produced NPs was tested against a mouse muscle cancer cell line model in a dose-dependent manner. CH-Alg/vit C was found to present potential anti-cancer activity, with a half-maximal inhibitory concentration (IC50) of 1.1 g /mL for vit C loaded into CH-Alg NPs.

Keywords: *Chitosan; alginate; nanoparticles; biocompatibility/biodegradability; vitamin C.*

INTRODUCTION

The incorporation of active ingredients into nanosystems to increase their shelf life, bioactivity, and bioavailability without inducing immune-system reactions has become a research hotspot [1, 2]. Nanosystems are able to deliver drugs to the right place, at the appropriate times, and at the right dose [3]. However, the nanoencapsulation process of sensitive active materials must be done under specific conditions such as high temperature, pH, and various levels of water/oil to avoid any damage inflicted to the active ingredient[4]. Nanoparticle (NP) delivery systems play important roles in increasing drug stability, prolonging the therapeutic period, and facilitating enteral and/or parenteral administration, which may minimize drug degradation and metabolism [5,6]. Many bases have been used to synthesize NPs, such as lipids that are the base of nanoliposomes, synthetic polymers, natural biopolymers, and polysaccharides [7]. Typically, liposomes are made from a lipid bilayer and are usually used as delivery systems for nutrients and drugs [8]. The main advantages of liposomes are biocompatibility and the ability to modify the surface toward amphiphilicity [9]. Conversely, the main disadvantage of standard liposome formulations is their rapid clearance from circulation by the reticuloendothelial system. To avoid this problem, polyethylene glycol molecules are attached onto their surfaces as a protective layer to increase their serum half-life [10]. Other limitations of liposomes are their rapid leakage of water-soluble drugs under unsuitable storage conditions [11]. Recently, NPs have been prepared from natural biodegradable polymers as a drug-delivery system. Among them, chitosan (CH)

and alginate (Alg) are encouraging and commonly used in the pharmaceutical industry to regulate drug releases [12]. Alg is a linear polysaccharide that is soluble in water, biodegradable, and biocompatible [13]. It has potential advantages in numerous pharmaceutical and biomedical applications such as drug delivery and cell encapsulation [14]. Earlier studies have reported that the gelling property of Alg can be used to create tiny gel bases containing small particles [15]. Thereafter, a neutral polymer such as poly-L-lysine (PLL) with a positive charge needs to be added to produce a polyectrolyte complex. However, PLL has toxic properties and may induce immune-system reactions. Accordingly, the linear cationic polymer CH, which is biodegradable, biocompatible, and nontoxic, has been used to replace PLL [13]. PLL addition also extends the duration of the active ingredients' existence, and improves absorption through the close junctions [16,17].

Vitamin C (ascorbic acid or ascorbate) is an elementary compound with important roles in several enzymatic reactions, such as collagen syntheses and antioxidant activities [18]. Reactive oxygen species (ROS) are constantly produced during common physiological proceedings, and can simply induce peroxidation in the lipids of membrane, leading to the accumulation of lipid peroxides [19]. ROS destroy important biomolecules such as lipids, nucleic acids, carbohydrates, and proteins [20]. Thus, if ROS are not successfully scavenged by cellular components, they cause diseases, and may interact in a cytotoxic manner with biological systems. ROS are known to cause cellular damage, aging process in prostate, colon cancers, and atherosclerosis, among others. Indeed, ROS are reportedly involved in more than 100 diseases [21, 22].

Antioxidants are composites that can interrupt or prevent the oxidation of lipids and/or other molecules by preventing the initiation or propagation of oxidative chain reactions [23]. Thus, they can increase shelf life by slowing down lipid peroxidation, which is one of the main causes of the deterioration of food and pharmaceutical products during manufacture and storage [24]. Accordingly, research on natural and safe antioxidant sources has increased in recent years [25]. Vit C for cancer treatment and prevention has also been given attention [26]. The beneficial properties of vit C for cancer through its antioxidant capacity have been studied [27] despite the poor bioavailability of vitamin C that may be due to its high solubility in water and inability to be stored in the body. Consequently, vit C is rapidly consumed in the case of any infection, and must be given in high dosages [26].

The current study aimed to incorporate vit C into CH-Alg NPs to improve the bioavailability of vit C, and enhance its activity as a supplement for cancer prevention and/or prevention at low concentrations. A nanocarrier was also developed that can undergo minimal degradation in the stomach, and be in a prolonged contact with the bioactive ingredients in the intestines.

MATERIALS AND METHODS

Materials

RPMI-1640 supplemented with 100 units/mL penicillin, 15% calf bovine serum, 100 μg/mL streptomycin, sodium alginate (Na-Alg), $CaCl_2$, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ascorbic acid, hydrochloric acid, and dimethylsulfoxide (DMSO) were purchased from (Sigma–Aldrich, USA). Trypsin-EDTA was purchased from US Biological (USA), and food-grade CH with 95% deacetylation (individual colonies with different morphology) was obtained from Paragon Specialty Products (LLC Rainsville, AL, USA).

Preparation of CH-Alg/vit C NPs

Na-Alg (3.0 mg/mL) and $CaCl_2$ (3.35 mg/mL) solutions were prepared by being dissolved in distilled water. Hydrochloric acid (HCl) was used to adjust the pH to 5.1. CH solution was prepared by dissolving 2.5 g of CH in 50 mL of (0. 25 M) HCl. CH-Alg (blank) was prepared according to Rajaonarivony's method [28] with modifications. Calcium alginate (Ca-Alg) in pre-gel phase was initially prepared by adding 2 mL of $CaCl_2$ to 10 ml of Na-Alg by continuous stirring for 30 min. Thereafter, 4 mL of CH (0.8 mg/mL) was added to the pre-gel, and stirred for an additional h.

In a typical procedure, CH-Alg was loaded with vit C by adding various amounts of vit C (0.32, 0.65, 1.3, and 2.6 g/mL; final volume of the preparation) to $CaCl_2$, and following the remaining steps as in the preparation of blank CH-Alg NPs. The resultant suspension was placed in an ultrasonic water bath to reduce the particle sizes of CH-Alg/vit C. Both blank and CH-Alg loaded with vit C were placed in an ultrasonic water bath, and sonicated at a low frequency 20 kHz for 60 min with stopping per 10 min, for 3 min at 25 °C.

Transmission electron microscopy (TEM) analysis

The morphology and size of CH-Alg/vit C were examined by TEM (model CM120, Phillips, Holland). A drop of sample was placed on a carbon-coated copper grid and stained with 1% of neutral phosphotungstic acid solution for 2 min. The excess stain was removed by wicking with filter paper, before the stained sample was analyzed.

Zeta-potential and particle-size measurements

The mean diameter and zeta potential of the CH-Alg/vit C NPs were tested by dynamic light scattering (DLS) by using a DLS system (Brookhaven NanoBrook 90 Plus, USA). The surface charge and particle-size distribution of NPs were determined with a Zeta Potential Analyzer (Brookhaven NanoBrook ZetaPlus, USA). DLS and zeta potential analyses were used to study the effects of pH on the size and zeta potential of the prepared CH-Alg/vit C, which were studied after being subjected to various pH values (3, 6 and 9). In a typical procedure, 100 µl of NP was dispersed in 1 mL of distilled water (DW). All sample measurements were run in triplicate, and the average values were reported.

1, 1-Diphenyl-picryl-hydrazyl (DPPH)-scavenging assay

To test the antioxidant activity of CH-Alg/vit C, the activity for scavenging the free radical DPPH was studied according to the Brand-William method (29). To prepare a stock solution of DPPH, 4 mg of DPPH was dissolved in 100 ml of methanol. The prepared stock solution was stored in darkness at 20 °C. The loaded CH-Alg/vit C NPs and the components alone as blank (CH and Alg) with various concentrations of vit C (0.32, 0.65, 1.3, and 2.6 g/mL) were prepared. DPPH solution alone was used as a negative control. Then, 1 ml of CH-Alg/vit C was mixed with 1 ml of DPPH solution. After conducting the measurement at 450 nm, the mixture was shaken, and incubated in darkness for 30 min at room temperature. Basically, a low absorbance indicated a high free-radical scavenging activity. Thus, DPPH-scavenging activity was calculated as follows: (%) = [(Ac-As)/Ac] × 100. In this equation, Ac is the absorption of DPPH solution alone, and as is the absorption of DPPH solution with CH-Alg/vit C.

Cell-viability assay

A mouse muscle cancer cell line was seeded at 1×10^4 cells/well with RPMI 1640, 15% FBS, and 1% penicillin–streptomycin, and then incubated for 24 h at 37 °C and 5% CO_2. When a confluent monolayer was achieved, the existing medium was removed and replaced with fresh medium with various concentrations of CH-Alg/vit C. Cell viability was measured after 72 h of exposure to the NPs, after which 28 µL of 2 mg/mL of MTT solution was added, and the mixture was incubated for 1.5 h at 37 °C. Wells were then solubilized with 130 µL of DMSO followed by incubation at 37 °C for 15 min with gentle shaking. After checking if the crystals were completely dissolved, the absorbance was determined on a microplate reader at 450 nm (test wavelength). All the assays were performed in triplicate (Organon Teknika Reader 230S, Austria).

RESULTS AND DISCUSSION

To the researchers' knowledge, this report has been the first one on the preparation of CH-Alg loaded with vit C at nanoscale to avoid problems related to inducing immune-system reactions, and improve penetration efficiency without the need to add a penetration enhancer (e.g., polymers). [30] utilized a similar system to enhance the penetration of drug nanocarriers. Additionally, all ingredients used in the preparation were natural, nontoxic, and inert; because they were all metabolized, and left the body without any probability of accumulation in body tissues after penetrating the target tissues. Conversely, other kinds of materials such as PEG can accumulate between or inside body tissues and may cause irritation [31].

Preparation of CH-Alg incorporated with vit C

Polyelectrolyte complexes (PECs) have been used as the base to prepare CH-Alg complexes and produce NPs. The underlying principle depended on the electrostatic interaction between negative and positive charges, thereby inducing spontaneous assembly at near-neutral pH. In the present work, the main composition of CH-Alg NPs was Na-Alg/$CaCl_2$/CH at 30:6.7:3.2 mass ratio [32], which was selected to produce a stable formulation in terms of separation into two layers and/or aggregation at room temperature for several days of Ca-Alg in pre-gel base. The amount of cationic polymer to form NPs was also appropriate. The main advantages of using CH to interact with Alg were the non-requirement of organic solvents and cross-linking chemical agents, thereby leading to the reduced toxicity and side effects. Accordingly, CH was used as an alternative agent to covalently cross-link hydrogels. According to the previous studies [33,34,35], the complexation between Alg and CH to form NPs offered a great facility, because they exhibited unique properties. The modified Rajaonarivony's method, which typically did not need the use of any organic solvents or synthetic organic compounds, was used. This method was inexpensive,

simple, rapid, and suitable for large-scale production. The known techniques to prepare NPs based on lipids and/or polymers involve the dissolution of one or more of their ingredients in organic solvents, which may disturb the chemical structure of the active ingredients. These substances have also been expensive and toxic in the final product [36, 37]. The preparation of vit C loaded into CH-Alg NPs was easy, because vit C was highly water soluble (hydrophilic). Thus, the successful entrapment of vit C was achieved by dissolving different concentrations of vit C (with negative charge) in DW prior to their incorporation into $CaCl_2$ (which is one of the most important compounds that produces Ca-Alg and has a positive charge). Thereafter, the CS solution was added, and a Tyndall effect was observed, thereby confirming the production of vit C in nanocrystal suspension. Basically, the gel phase provided a suitable base medium in which Alg, Ca ions, and polymer can interact to produce NPs [38]. Thus, when CH was incorporated into the Ca-Alg in pre-gel phase (which already had vit C nanocrystals), Ca-Alg existence in the discrete form would simultaneously entrap vit C existing in the medium. Hence, the final product was consisted of a suspension of CH-Alg NPs containing vit C nanocrystals entrapped within CH molecules.

Morphological Characterization

Morphology analysis of CH-Alg incorporated with vit C was conducted by TEM. The results showed that the CH-Alg NPs were spherical, and had sizes ranging from 25 nm to 30 nm, as shown in Figure (1), after being subjected to ultrasonication. The obtained CH-Alg NPs were within the same range of the sizes obtained in a previous study (20–50 nm), and involved the same principle as that of loaded nifedipine in CH-Alg NPs [32]

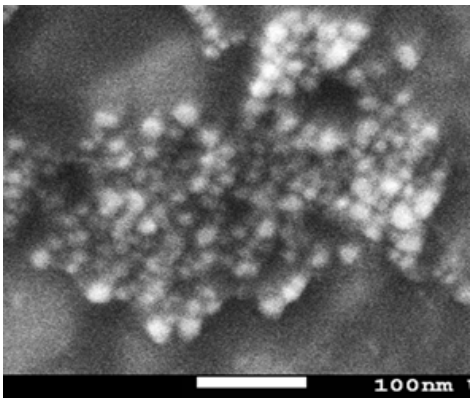
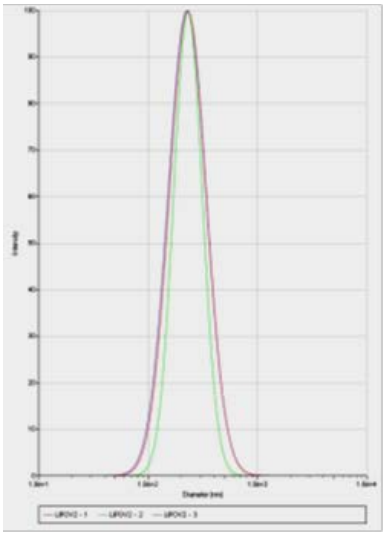


Figure 1. TEM image of vit C loaded into CH-Alg NPs

DLS and zeta potential

DLS was used to characterize the average size of CH-Alg/vit C ,and measure their zeta potential values (Table/Figure). The DLS-derived sizes were 229 nm, although the finding of larger sizes by DLS compared with TEM has been well known**.** Moreover, zeta potential analysis was used to characterize the stability and dispersion of CH-Alg NPs incorporated with vit C. Zeta-potential values presented a negative charge ranging between -34 and -43 (Figure 2). These values indicated that the CH-Alg prepared in the present study had uniform particle size, were stable, and had a high negative zeta potential that may prevent aggregation. According to a previous report [39], zeta-potential values of ≤30 mV or ≥30 mV reflected the stability of the materials. The high negative charge may be explained by the carboxyl groups in the Alg chain, because the analysis was performed in the neutral aqueous solution. Under this condition, no chance to protonate the amine groups in CH existed, and somehow the negative charge of carboxyl groups in Alg was presented on the surface. Additionally, the results showed that the polydispersity values, which are important indicators of particle aggregation, were between 0.109–0.15 for NPs loaded with Eos, and 0.3 for empty NPs [40]. These values indicated that the preparation was monodisperse, and had a minimum tendency for aggregation.



Sample ID	Zeta Potential (mV)	Mobility (µ/s)/(V/cm)	Frequency (Hz)	Frequency Shift (Hz)
LIPOV2 - 1 @ 24.0 °C	-42.31	-3.25	220.91	-30.38
LIPOV2 - 2 @ 24.0 °C	-26.61	-2.04	231.74	-18.26
LIPOV2 - 3 @ 24.0 °C	-46.22	-3.55	219.96	-31.33
LIPOV2 - 1 @ 25.0 °C	-28.64	-2.24	229.84	-20.16
LIPOV2 - 2 @ 25.0 °C	-28.85	-2.25	229.84	-20.16
LIPOV2 - 3 @ 25.0 °C	-36.74	-2.87	225.68	-26.57
Mean:	-34.90	-2.70	226.33	-24.48
Std Err:	3.32	0.25	2.03	2.32
Std Dev:	8.14	0.61	4.98	5.69

Sample ID	Eff. Diam. (nm)	Polydispersity	Baseline Index
LIPOV2 - 1	230.21	0.165	5.5
LIPOV2 - 2	231.21	0.096	7.1
LIPOV2 - 3	226.59	0.172	7.9
Mean:	229.34	0.144	6.8
Std Err:	1.40	0.024	0.7
Std Dev:	2.43	0.042	1.2

Figure 2. DLS and zeta potential of CH-Alg loaded with vit C

Effects of pH on size and charge

Typically, pH plays a very important role in the preparation of colloidal formulations. In the current study, blank and CH-Alg/vit C were subjected to different pH values (3, 6 and 9). DLS was also used to study this effect on CH-Alg/vit C. Figure 3 shows the effect of pH on the size (as measured by DLS) of as-prepared CH-Alg/vit C. At pH 3, the size of the CH-Alg/vit C formulations were around 350 nm (tendency to aggregate). With increased pH near the basic end until reaching pH 9, the formulations were within the micrometer range. These results can be explained by the fact that the interaction of both Alg and CH at pH < 5.0 caused the increased particle size and particle aggregation. Aggregation occurred when the CH solution's pH was >6.0 (data not shown) owing to the lost solubility of CH, given that the pKa value of CH was 6.5. According to an early report [34], the optimum size distributions were achieved at pH 5.0–6.0 and 4.0–6.4 of CH and Alg solution, respectively; thus, pH 6.0 was selected in this study to prepare CH-Alg NPs with small sizes. At this pH, the carboxyl groups (COO) of Alg were also ionized and the amine groups (NH_3) of CH were protonated, so it was considered the optimum pH of the interaction to produce the polyionic complex formula.

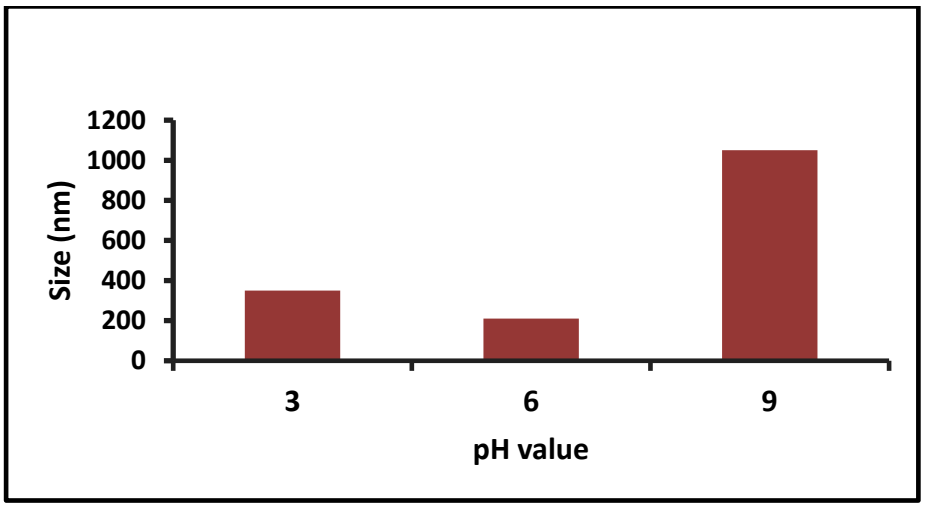


Figure 3. DLS analysis of the effects of various pH values on the size of CH-Alg/vit C NPs

Zeta potential was further used to study the behavior of surface charge at various pH values. At neutral to basic pH, the zeta potential of NPs presented a negative charge. Accordingly, the produced NPs presented a negative particle surface, because the test was conducted in DW. Meanwhile, at acidic pH, the NPs presented a positive charge that

resulted from the ionization of amine groups in the CH molecules (data not shown), the same results were obtained by [32].

Notably, the zeta potential of the blank was almost the same as those of the loaded preparation, which was attributed to CH and Alg having no effect on the zeta potential in the presence of vit C that was bound with $CaCl_2$. Conversely, a slight difference was observed between the sizes of the blank and CH-Alg/vit C. The ability of CH to protonate its amine group and its tendency for deacetylation in acidic to neutral solutions can explain its solubility in acidic solutions [41]. Notably, the protonated amine group of CH in acidic to neutral solutions has been the key to its bioadhesiveness and negative charge, such as to the mucosal membrane [42]. This important feature of CH enabled the preparation to achieve one of the most important aim of developing nanocarriers, i.e., nondegradation in the stomach, and prolonged contact time with the bioactive ingredient in intestinal epithelia, as well as the increased absorption through the close junctions and into the paracellular pathway in neutral and alkaline pH environments.

Antioxidant potential study

A positive DPPH-scavenging test showed a purple color after dissolution in methanol. When DPPH reacted with an antioxidant composite, DPPH was reduced by donating hydrogen or free electrons. Consequently, the decolorization degree reflected the scavenging action of the loaded NPs. Figure 4 displays the DPPH-scavenging ability of the loaded NPs in a dose-dependent manner. Meanwhile, the antioxidant activity of CH-Alg before loading with vit C (negative control) was the same as the antioxidant activity of DPPH solution without the sample, which indicated that the antioxidant activity originated from the activity of vit C incorporated into CH-Alg NPs. Furthermore, despite the fact that vitamin C is a well-known antioxidant, it displayed too low DPPH-scavenging activity in free form but showed a significant DPPH-scavenging activity at the same concentration in encapsulated form (Figure 4). Thus, the antioxidant activity of vit C was enhanced after loading into CH-Alg NPs. The same result was reported upon the encapsulation of vit C by fatty acid micelle, and the encapsulation of vitamin C in the form of NutraNanoSpheres™ [43].

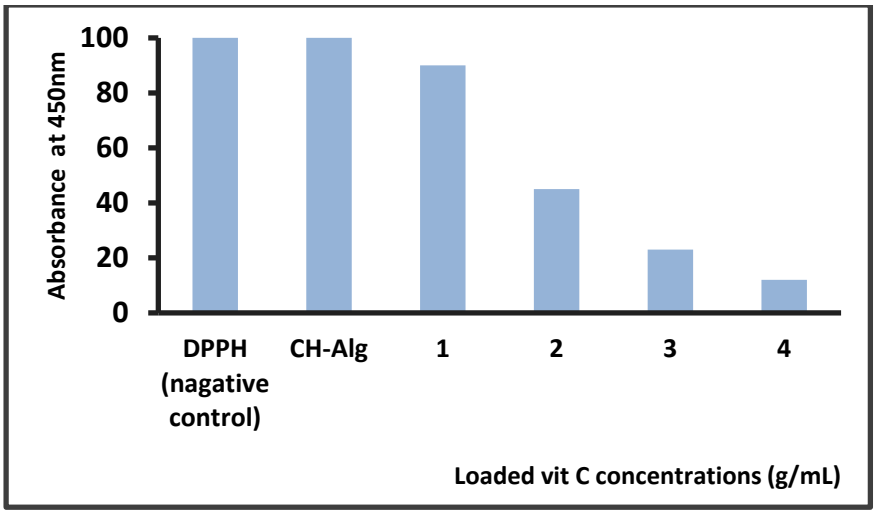


Figure 4. Total antioxidant activities of different concentrations of vit C (0.32, 0.65, 1.3, and 2.6 g/ml, represented as 1, 2, 3, and 4 respectively) loaded into CH-Alg NPs, and the negative control of DPPH and CH-Alg.

Anticancer activity of the prepared conjugated NPs

NPs can avoid potential problems in drug delivery such as inducing immune-system responses, which is well known to occur with large particles. Nanoliposomes are reportedly better systems for the delivery of drugs and bioactive ingredients than their larger counterparts. Herein, the anticancer activity was evaluated depending on the principle of DPPH-scavenging assay, which depended on measuring the decolorization degree after interaction with antioxidants. Through this approach, the antioxidant activity of vit C in free form was compared with the same concentration and after loading with CH-Alg NPs. Figure 5 shows the percentage of inhibited cells treated with CH-Alg NPs incorporated with various concentrations of vit C. CH-Alg NPs loaded with vit C presented antioxidant activity against mouse muscle cancer cell lines in a dose-dependent manner. The ability of CH-Alg/vit C to inhibit

the viability of mouse muscle cancer cells was significantly enhanced compared with the same concentration of vit C in the free form. In this study, various concentrations of vit C (0.325, 0.65, 1.37, and 2.74 g/mL) were incorporated into CH-Alg NPs. Among these concentrations, 1.37 g/mL was used for the subsequent experiments on the stability of CH-Alg/vit C solution (stable without separation into two or more layers). The half-maximal inhibitory concentration (IC50) of vit C loaded into CH-Alg NPs was found to be 1.1 g /mL.

Overall, the results showed that the primary goal of the present study, namely, to develop water-soluble combinations of a nanocarrier system to deliver cancer drugs and/or supplements by using biocompatible biodegradable ingredients (CH and Alg) and thus enhance the antioxidant activity of vit C at low concentrations, was reached. This result was expected because of the low concentrations of vit C incorporated in nanoscale. The findings of this study were also consistent with those obtained using an antioxidant method in test tubes that showed the antioxidant activity of various concentrations of vit C loaded into CH-Alg NPs in a dose-dependent manner.

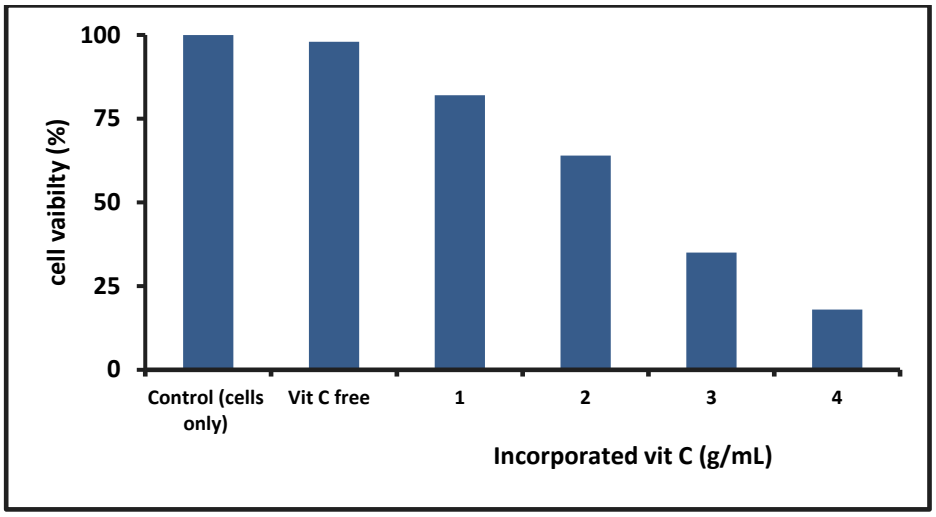


Figure 5. Antioxidant activities expressed as the percentage of inhibited cell growth by using different concentrations of vit C (0.32, 0.65, 1.3, and 2.6 g/ml, represented as 1, 2, 3, and 4 respectively) loaded into CH-Alg NPs and the free form of vit C in relation to a control (cancer cells alone).

CONCLUSION

This study has reported the entrapment of vit C within CH/Alg NPs for the first time. All the ingredients used to prepare NPs were natural. The final preparation showed a great control of vit C degradation in the stomach and intestines. A major conclusion of this study was that the anticancer activity of vit C at low concentrations can be enhanced by entrapping it within CH-Alg NPs.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Zystein, LLC (Fayetteville, AR, USA) for providing some of the natural reagents and chemicals used in this study. This research was partially supported by Baghdad University (Baghdad, Iraq) and University of Arkansas in Fayetteville, AR. USA.

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