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Cytotoxicity of Silica Nanoparticles with Transcaucasian Nose-Horned Viper, *Vipera ammodytes transcaucasiana*, Venom on U87MG and SHSY5Y Neuronal Cancer Cells

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Abstract Highly bioactive compounds of the snake venom make them particular sources for anticancer agent development. They contain very rich peptide-protein structures. Therefore, they are very susceptible to environmental conditions such as temperature, pH, and light. In this study, *Vipera ammodytes transcaucasiana* venom was encapsulated in PAMAM-G4 dendrimer by sol-gel method in order to prevent degradation of venom contents from the environmental conditions. For this purpose, nanoparticles were prepared by sol-gel methodology and SEM analyses were performed. U87MG and SHSY5Y neuronal cancer cell lines were treated with different concentrations of venom-containing nanoparticles and cytotoxicity was determined by MTT assay. IC_{50} values of nanoparticles with snake venom were calculated as 37.24 and 44.64 μg/ml for U87MG and SHSY5Y cells, respectively. The IC_{50} values of nanoparticles with snake venom were calculated as 10.07 and 7.9 μg/ml for U87MG and SHSY5Y cells, respectively. As a result, nanoparticles with *V. a. transcaucasiana* venom showed remarkably high cytotoxicity. Encapsulation efficiency of nanoparticles with 1 mg/ml snake venom was determined as %67 via BCA™ protein analysis. In conclusion, this method is found to be convenient and useful for encapsulating snake venom as well as being suitable for drug delivery systems.

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Introduction

Transcaucasian nose-horned viper, *Vipera ammodytes transcaucasiana*, is a venomous snake endemic mostly in northern Anatolia and in some parts of Georgia [1]. Recently, the species have also been recorded from more inland Anatolia [2, 3]. Snake venom is a complex mixture of enzymes, peptides, carbohydrates, minerals, and proteins of low molecular mass with specific chemical and biological activities. Components of snake venoms can be used in the treatment of cancer, arthritis, thrombosis, multiple sclerosis, pain, neuromuscular disorders, blood and cardiovascular disorders, infections, and inflammatory diseases [4–8]. Recently, highly bioactive compounds of the snake venoms make them unique sources for anticancer agent development and there are many studies that focus on this subject [3, 4].

Sol-gel method used in this study is formed from the colloidal suspension. This process consists of the production of inorganic matrix, followed by the gelation of the sol and dry gel (xerogel) [8]. Sol-gel process involves the formation of mineral phases starting from soluble molecular precursors, following an inorganic polymerization reaction. The reaction is at room temperature, in water or organic solvents and in a wide range of pH/ionic strength conditions [5, 9]. A series of sequential chemical reactions is formed by electrochemical interactions of the particles with surface charge at certain temperature [10]. In this method, particles come together to form a network structure. This network structure interconnects to form the final network structure. Polymer molecule formed prolongs as a result of cross-linking which ends up with the solidification in solution [8].

In this study, *Vipera a. transcaucasiana* venom was encapsulated with PAMAM-G4 dendrimer by sol-gel method. Encapsulation efficiency of nanoparticles was performed by protein and cytotoxicity assays. After encapsulation process; efficiency assay, SEM analysis, and cytotoxicity analysis of these nanoparticles were performed.

Materials and Methods

Materials

Lyophilized *Vipera a. transcaucasiana* crude venom was provided by Bayram Göçmen from the previous study. BCA™ Protein Assay Kit was purchased from Thermo Scientific (Waltham, Massachusetts, USA). PAMAM-G4 dendrimer, tetraethyl orthosilicate (TEOS), Tris-HCl, Folin's reagent, phosphate buffer saline (PBS), sodium carbonate (Na_2CO_3), hydrochloric acid (HCl), ethanol (C_2H_5OH), and MTT assay kit were obtained from Sigma Aldrich (Missouri, USA). Ultra-pure water was from an in-house nano-pure water system (Sartorius, Germany). DMEM Ham's F12, fetal bovine serum (FBS), penicillin/streptomycin, and dimethyl sulfoxide (DMSO) were provided commercially (Lonza, Visp, Switzerland) for in vitro cell culture studies.

Preparing Nanoparticles by Sol-Gel Method

Tetraethyl orthosilicate (TEOS) were used as silica source in the production of aerogels. Eight hundred forty-four microliters of ultra-pure water, 100 μL of HCl (0.1 M), and 56 μL of TEOS

(0.25 M) were stirred for 15 min in order to get TEOS hydrolysis solution and then homogenized by vortexing.

Trizma was used to solve PAMAM dendrimer solution. Trizma solution (0.5 M Tris-HCl) was prepared by dissolving the 61 g Trizma mania in 1000 L of ultra-pure water (pH 7.6). Dendrimers of the PAMAM was provided by dissolving Trizma in 10× dilution.

PAMAM solution (50 μL), TEOS solution (100 μL), and snake venom-PBS solution (850 μL) were stirred in order to encapsulate snake venom via sol-gel method by using a magnetic stirrer with a low speed for 1 h. The gel mixture was centrifuged for 15 min at 1000 rpm at 37 °C for the separation of the formed particles after encapsulation. Sub-phase of the solution was removed and the upper phase was washed with ultra-pure water by the repeating same process for three times after the centrifugation step.

Protein Assay

Protein concentration was determined from diluted crude venom and nanoparticle samples (1 mg/mL) in deionized water by BCA™ Protein Assay using a UV/V spectrophotometer (VersaMax, Molecular Devices, CA, USA) at a wavelength of 562 nm. Bovine serum albumin was used as a reference.

Efficiency of encapsulation was determined with BCA™ Protein Assay by using the formula below:

$$\%\text{Efficiency} = \Big[\text{Total protein before encapsulation}(\mu\text{g/ml}) - \text{Total protein after encapsulation}\ (\mu\text{g/ml})\Big] / [\text{Total protein after encapsulation}]$$

SEM Analysis

SEM analysis was performed with a scanning electron microscope (Quanta 250 SEM) at Izmir Institute of Technology. The humidity of the sample to be analyzed was removed by lyophilization apparatus. Examples were placed on a plate with heights equal to a maximum of 17 mm. The analyses of samples were performed at different magnifications.

In Vitro Cell Culture Studies

U87-MG and SHSY5Y cells were cultivated in Dulbecco's Modified Eagle's medium F12 (DMEM/F12), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2. The cells were passaged twice a week and were used in the test actively proliferating cells at the exponential phase.

Morphological Studies

The morphological studies of the cells were performed with an inverted microscope (Olympus, Tokyo, Japan) compared to the control group 48 h after treatment.

Cytotoxicity Assay

Cytotoxicity of crude venom and nanoparticules were evaluated with venom on U87MG and SHSY5Y cells by MTT assays [11]. Cell lines were purchased from ATCC (Manassas, VA, USA). Cell viability was determined after 48 h with MTT assay. The best cytotoxic concentration was determined after calculating the level of U87MG and SHSY5Y cell viability. For this purpose, cells (1×10^5cells/ml) were cultivated in DMEM-Ham's/F12 medium into 96-well plates for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. After 24 h, cells were treated with different concentrations of snake venom (0.4, 4, and 40 μg/ml) and incubated for 48 h. MTT (2.5 mg/ml) stock solution was added as 25 μl at the end of each incubation period and incubated for 4 h at 37 °C. Dissolved formazan crystals in DMSO were measured at 570 nm (reference filter 630 nm) with a UV visible spectrophotometer.

Percentages of surviving cells in each culture were determined by the following formula:

$$\%\text{Viable cells} = [(\text{absorbance of treated cells}) - (\text{absorbance of blank})] / [(\text{absorbance of control}) - (\text{absorbance of blank})] \times 100$$

Statistical Analyses

Statistical analysis was conducted by ANOVA followed by Tukey's test for comparisons between groups. 0.05 was taken as the p value to determine statistical significance ($p < 0.05$).

Results and Discussion

Protein Assay

The snake venom encapsulation efficiency in PAMAM G-4 dendrimer with sol-gel method was estimated by measuring total protein amount. Total amount of protein was determined with BCA™ Protein Assay Kit and encapsulation efficiency was calculated with the value obtained before and after the process. According to the protein analysis, encapsulation efficiency was determined as %67 after 1 mg/ml snake venom encapsulation.

Venkatesan et al. [12] encapsulated Russell's viper snake venom with nanoparticles by using chitosan tripolyphosphate. The efficiency of encapsulation was found to be close to %70 [12]. Data obtained from Venkatesan et al. [12] on venom encapsulation efficiency strongly correlates with our study which was based on encapsulation via PAMAM G-4 dendrimer by using sol-gel method.

SEM Analysis

Nanoparticles, snake venom, and nanoparticles containing snake venom were analyzed with scanning electron microscopy (SEM). The results of the analysis are shown in Fig. 1. Nanoparticles imaged with SEM are generally found to be round in shape and bonded together. The nanoparticles were observed to form aggregates. Free nanoparticles at high magnification are hardly seen apart due to the aggregation of particles. General views are not selected at low magnification. Nanoparticles are found to be more distinct at higher magnifications. The crystal structure of snake venom can be selected substantially in SEM analysis at 2500×

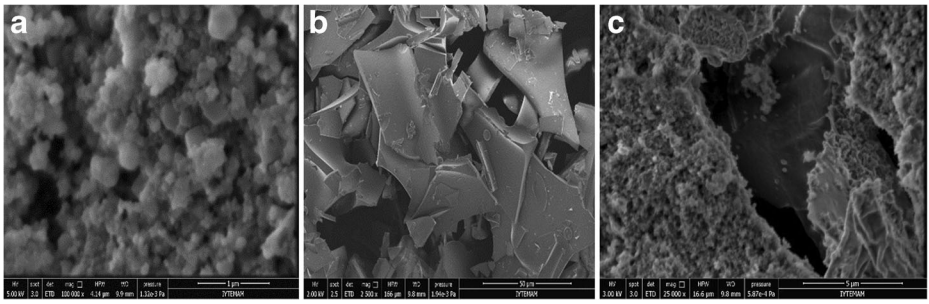

Fig. 1 Scanning electron microscopy (SEM) images of the samples. **a** Free nanoparticles. **b** Only snake venom. **c** Nanoparticles containing snake venom

magnification. Snake venom-containing nanoparticles are in unprecedented crystalline appearance outside the capsule demonstrating the capability of the capsulation efficiency.

In Vitro Cell Culture Studies

Snake venom, nanoparticles, and nanoparticles containing snake venom were tested on U87MG and SHSY5Y cells for in vitro cell culture studies. The effect of venom and nanoparticles on the morphology of the cells was observed by using inverted microscopy after 48 h. Venom exposure is demonstrated in Figs. 2 and 3. The venom decreased the viability of U87MG and SHSY5Y cells at lower dose (4 μg/ml) when compared to the nanoparticles containing snake venom. Nanoparticles with venom showed cytotoxicity at 40 μg/ml dose on U87MG and SHSY5Y cells which can be explained by the slow release of snake venom upon encapsulation with nanoparticles.

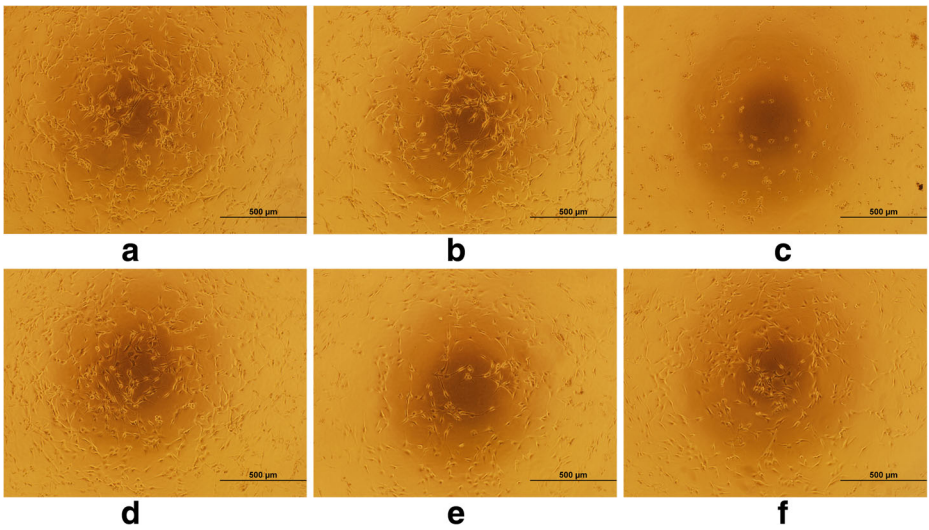

Fig. 2 Dose-dependent venom induced morphologic changes of U87MG cells viewed by inverted microscope for free nanoparticle, *V. a. transcaucasiana* venom and nanoparticles with venom 48 h after treatments. **a** Untreated U87MG cells. **b** 4 μg/ml venom. **c** 40 μg/ml venom. **d** Nanoparticles with 4 μg/ml venom. **e** Nanoparticles with 40 μg/ml venom. **f** Free nanoparticles (40 μg/ml)

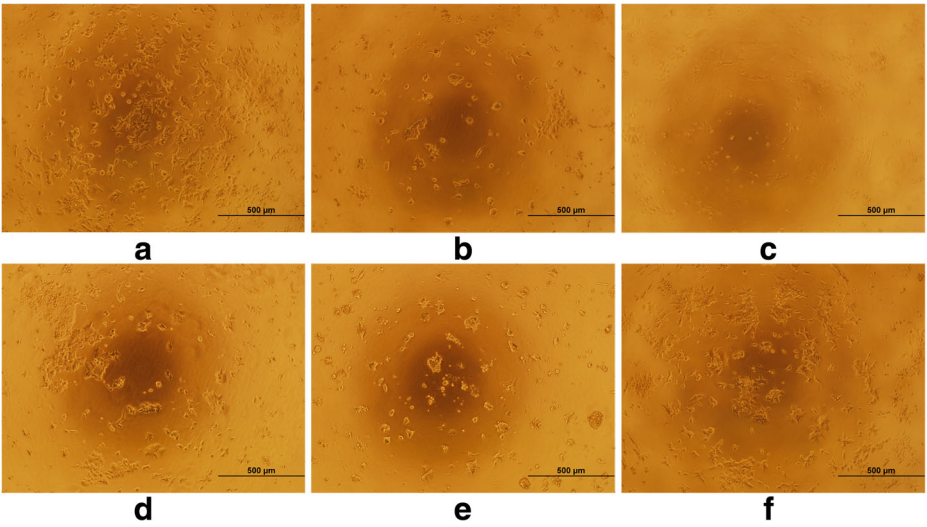

Fig. 3 Dose-dependent venom induced morphologic changes of SHSY5Y cells viewed by inverted microscope for free nanoparticle, *V. a. transcaucasiana* venom and nanoparticles with venom 48 h after treatments. **a** Untreated SHSY5Y cells. **b** 4 µg/ml venom. **c** 40 µg/ml venom. **d** Nanoparticles with 4 µg/ml venom. **e** Nanoparticles with 40 µg/ml venom. **f** Free nanoparticles (40 µg/ml)

Cytotoxicity Assay

Cytotoxicity of nanoparticles and snake venom on U87MG and SHSY5Y cells were detected with MTT assays. In Fig. 4, it can be seen that crude venom of the *V. a. transcaucasiana* and

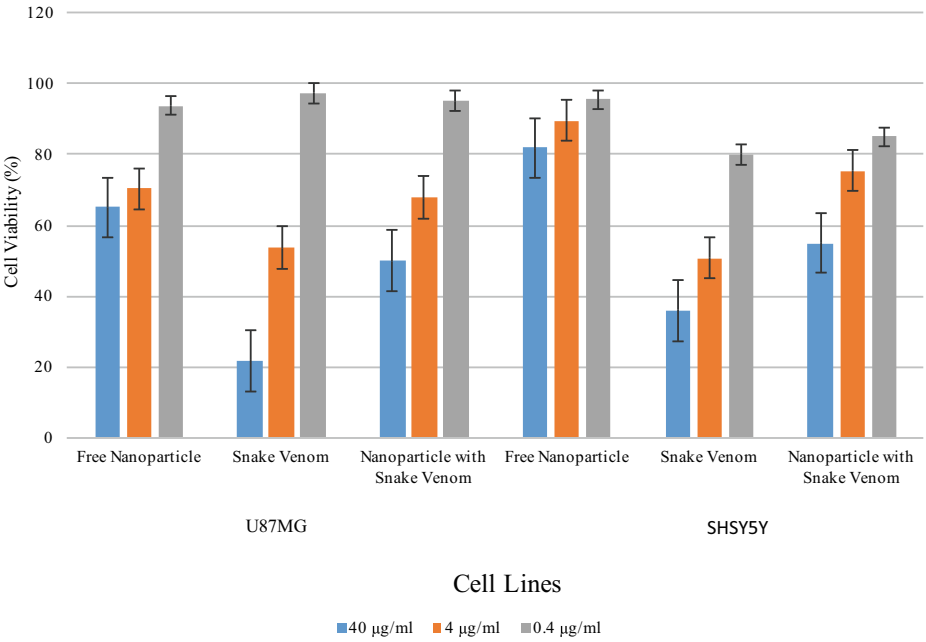

Fig. 4 Viability of cells 48 h after the treatments free nanoparticles, snake venom, and nanoparticles containing snake venom was applied to the cells with different concentration (40, 4, and 0.4 µg/ml)

Table 1 IC_{50} values on U87MG and SHSY5Y cell lines following free nanoparticle, *V. a. transcaucasiana* venom and nanoparticles with venom treatment for 48 h

IC_{50} (μg/ml)	U87MG	SHSY5Y
Free nanoparticle	–	–
Snake venom	10.07 ± 1.12	7.90 ± 1.22
Nanoparticles with snake venom	37.24 ± 2.04	44.64 ± 2.36

nanoparticles inhibits cell viability in a dose-dependent manner. According to the MTT results, *V. a. transcaucasiana* venom has remarkable cytotoxic effect on U87MG and SHSY5Y cells with 10.07 and 7.99 μg/ml after 48-h incubation, respectively.

In a study of Venkatesan et al. [12], Catla heart cell viability was determined as %87 after treatment with 5 mg/ml concentration of nanoparticles with Russell's viper snake venom by MTT assay. These results show that encapsulated snake venom with chitosan tripolyphosphate have high efficiency, but the release of snake venom from nanoparticles with this method is found to be slow. According to the results obtained, Russell's viper snake venom nanoparticles have not shown any cytotoxic effect when compared to nanoparticles used in our study [12]. The half maximal inhibitory concentration (IC_{50}) results of the nanoparticles, snake venom, and nanoparticles containing snake venom are given in Table 1. Results show that nanoparticles' IC_{50} results are much higher than the snake venom and nanoparticles containing snake venom showing free nanoparticles have very low inhibitory effect on U87MG and SHSY5Y cells. IC_{50} values of nanoparticles containing snake venom treatment on U87MG and SHSY5Y cells are determined as 37.24 and 44.64 μg/ml after 48 h. Based on the cytotoxicity results, the crude snake venom has much more inhibitory potency than the nanoparticles on U87MG and SHSY5Y cells which exhibit encapsulation efficacy. According to the literature, there are too many studies about cytotoxicity of effective silica nanoparticles with snake venom on different cell lines [12–18]. Badr et al. [4] found out that the combination of snake venom with silica nanoparticle was very cytotoxic with lower IC_{50} values when compared to the snake venom-only group. These results differ from our study due to the differences in methods and materials used for encapsulation process. Badr et al. [4] used TEOS and SiO_2 to form double mesoporous core-shell silica sphere and showed that using PAMAM-G4 slowed down the active ingredient release by sol-gel method [3]. Jamunaa et al. [6] discovered the differences in protein profiles and cytotoxicity of Southeast Asian snake venoms on different cell lines. They found the IC_{50} values between 1.4 and 51.9 μg/ml on MDCK or Vero cell lines [6]. Our prior studies on Turkey viper snake venoms also exhibited considerable cytotoxic effect on cancer cells (0.5–50 μg/ml). The results obtained in this study corroborated with the results in the above-mentioned studies.

Conclusion

In this study, the sol-gel method with PAMAM G-4 dendrimer was used for the first time in the preparation of free nanoparticles and *V. a. transcaucasiana* venom encapsulation. For this purpose, *V. a. transcaucasiana* venom was encapsulated by PAMAM G-4 dendrimer by using sol-gel method. This is the first study in literature about sol-gel application and *V. a. transcaucasiana* venom together. This study would provide data for new studies on clinical and pharmaceutical areas. Nanoparticles and snake venom-containing nanoparticles were analyzed with SEM and MTT assays. The results show that the snake venom nanoparticles would be a good candidate for

effective drug formulation in medical and pharmaceutical areas. In conclusion, *V. a. transcaucasiana* venom is found to be very effective on U87MG and SHSY5Y neuronal cell lines and sol-gel method is very useful for encapsulation of the *V. a. transcaucasiana* venom. This method is applicable for drug targeting, cancer therapy, and treatment. Silica nanoparticles with *V. a.* transcaucasiana venom have a large potential for development of drug delivery systems.

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