Synthesis of nano encapsulated low-molecular weight anticoagulants

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Received January 31, 2019; Accepted for publication March 15, 2019

Abstract

Several studies indicated that snake venoms were mainly peptides and proteins. Most of these substances have an impact on nervous system, while a number of toxins causing blood clotting disorders is not so great. Earlier we have isolated from the scorpions *Heterometrus laoticus* venom adenosine (Ado) and two dipeptides LeuTrp (LW) and IleTrp (IW) which demonstrated strong anticoagulant activity. To increase their stability and prolong anticoagulant effect, we have incorporated these compounds into nanomaterials. Two types of nanomaterials were used, one was thermosensitive nanogel composed of heparin and pluronic P123 (Hep-P123) and other one - complex of PAMAM dendrimer G3.0 and sulphated polysaccharide fucoidan (G3.0-Fu). Four nano encapsulated anticoagulants were prepared - Hep-P123-IW, Hep-P123-Ado, G3.0-Fu-IW, and G3.0-Fu-Ado. Their characteristics were characterized by Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Zeta Potential Measurement (ZPM). Loading efficiency of anticoagulant was evaluated by UV spectroscopy. TEM images showed that G3.0-Fu nanoparicle size increased from 32.35±5.09 nm to 46.46±5.38 nm and 72.76±10.93 nm when loaded with Ado and IW, respectively. Drug entrapmentefficiencies were 61.56 % for G3.0-Fu-Ado and 95.63 % for G3.0-Fu-IW. The TEM data for Hep-P123 nanogel demonstrated the decrease in nanoparticle size from 74.26±9.97 nm to 31.42±10.94 nm and 27.2±6.97 nm after loading with IW and Ado, respectively. Drug entrapment efficiencies were 69.76 % and 95.66 % for Hep-P123-Ado and Hep-P123-IW, respectively. DLS measurements when compared to TEM showed the larger nanoparticle size for all preparation. Interestingly, in contrast to TEM, DLS demonstrated the increase in the size for Hep-P123 nanogel after incorporation of IW and Ado. ZPM demonstrated increase in the potential for the anticoagulant loaded nanomaterials. The change in zeta potential was consistent with drug entrapmentefficiencies as well as with the result of TEM and DLS. We suggest that electrostatic interactions are responsible for holding Ado and IW inside the nanoparticles. The encapsulation of anticoagulants into nanomaterials may protect them from degradation under the biological environment, increase their availability in vivo and thus prolong their biological effects. Considering high biocompatibility of nanomaterials used, the encapsulated anticoagulants have a good potential for biomedical application.

Keywords. Anticoagulant, adenosine, PAMAM G3.0, Fucoidan, Heparin, Pluronic P123.

1. INTRODUCTION

Nanomaterials have been exploiting in various fields from basic researches to practical applications. Expecially, several kinds of these platforms played a vital role in drug delivery systems. For example, Drugs, bioactive molecules and proteins exhibited an improved therapeutic effect in biodegradable polymeric nanoparticles.[1-5]

Scorpion venom represents a mixture of substances which are mainly peptides and proteins and have a major impact on the function of the nervous system. Their main task is to paralyze a prey quickly. In addition to the neurotoxic compounds, scorpion venoms containtoxins inducing blood clotting impairment. In particular, the time clotting was increased 2.5 times and 2.3 times by the venous of *Pandinus imperator*and *Parabuthus transvaalicus*, respectively, while other venoms extended the coagulation time from 0.8 to 2 times.[6] The scorpion *Tityus discrepans* venom and its fractions affected clotting parameters as well.[7] A polypeptide inducing a thrombus formation in vivo and in vitro was isolated from Chinese scorpion *Buthus martensii Karsch* venom;[8] this polypeptide is the major component of the Chinese scorpion venom. From venom of *Tityus discrepans, a*nother isolated polypeptide has molecular weight about 6 kDa exhibiting

antifibrinolytic activity.[9] Our investigation of the scorpion *Heterometrus laoticus* venom showed that it contained components affecting the process of blood clotting.[10,11] In a further study, we isolated substances possessing anticoagulant activity and determined their structures. These were dipeptides LeuTrp (LW) and IleTrp (IW) as well as adenosine.[12,13]

To increase the biological effect of the isolated substances, we have encapsulated them into nanomaterials. Thermosensitive nanogel composed of heparin and pluronic P123 (Hep-P123) as well as complex of PAMAM dendrimer G3.0 and sulphated polysaccharide fucoidan (G3.0-Fu) were used as nanomaterials. Four nano encapsulated anticoagulants were prepared - Hep-P123-IW, Hep-P123-Ado, G3.0-Fu-IW, and G3.0-Fu-Ado. This paper describes the synthesis of the encapsulated anticoagulants.

2. EXPERIMENTAL SECTION

2.1. Materials

Fucoidan (Fu) and Adenosine (Ado) were from Sigma (USA). PAMAM dendrimer G3.0 and Hep-P123 were prepared at the Institute for Applied Materials Science as described.[14] Dipeptide IW was prepared as in.[13]

2.2. Synthesis of G3.0-Fu-IW and G3.0-Fu-Ado

Fu (25 mg) was dissolved in 2.5 ml water at room temperature. To the solution obtained, 1.68 mg IW in 0.08 ml water or 2.5 mg Ado in 0.04 ml water were added and stirred for 15 minutes (250 rpm) at room temperature. Then 25 mg PAMAM G3.0 dissolved in 2.5 ml water were added slowly and stirred for 15 minutes under the same conditions. To remove unincorporated IW or Ado, the reaction mixtures were dialyzed against distilled water. Finally, the dialyzed products were freeze-dried and stored for subsequent experiments.

2.3. Synthesis of Hep-P123-IW and Hep-P123-Ado

Hep-P123 copolymer (25 mg) was solubilized in 2.5 ml water at 15 ºC. To the solution obtained, 1.68 mg IW in 0.08 ml water or 2.5 mg Ado in 0.04 ml water was added into copolymer solution understirring at 15 ºC for 20 minutes. To remove unincorporated IW or Ado, the reaction mixtures were dialyzed against distilled water. Finally, the dialyzed products were freeze-dried and stored for subsequent experiments.

2.4. Morphology and size distribution of the nanoparticles

The size distribution was measured at Institute of Applied Material Science using SZ-100 machine. The scattered light was received at two angles, 90º or 173º. Stokes-Einstein equation was used to calculate particle size.

Morphology of nanoparticles were showed by TEM at VNU-University of Technology. 1µl sample solution was dropped on a net frame and let it dry naturally before dipped into uranyl acetate 1 % w/v solution and shake gently. Sample measurement was conducted on TEM (JEM-1400 JEOL) at 25 ºC.

2.5. Zeta Potential Measurement

The surface charge of nanoparticles was analyzed by SZ-100 instrument. Samples are injected into the disposable AGILE carbon cell. The result of zeta measurements is calculated from the move electrophoresis potential of the particle system.

2.6. Determination of anticoagulant content in nanomaterials by UV spectroscopy

The IW and Ado entrapment efficiencies and drug loading in the nanoparticles were determinedby the UV spectroscopy using Agilent 8453 UV-Vis Spectrophotometer. To calculate concentration, the adsorbtion at 280 and 260 nm was used for IW and Ado, respectively.

To make a calibration curve, the pure IW or Ado were dissolved in distilled water at concentrations of 1, 2.5, 5, 7.5 and 10 µg/mL. The relation between the concentration of IWor Ado and solution optical density is linear and described by equation $y = ax + b$, in which y and x are asigned with the optical density and concentration, respectively.

5 mL of each sample was added into a dialysis membrane (MWCO ~ 3500 Da. The bag was suspended in a beaker with 500 mL distilled water which was placed on a heater and stirred at 37±1 ºC, 50 rpm for 20 minutes. After that to determine the amount of free IW or Ado, the optical density of the solution in the beaker was measured. The amount ofIWor Ado encapsulated in the carrier system was calculated as a difference between the total quantity of anticoagulant used for synthesis and a free one in the beaker.

2.7 Statistical analysis

Statistical analysis was performed using ORIGIN 8.5.1 (OriginLab Inc., Northampton, MA, USA). All

experiments in this study were carried out at least three times, and the data are represented as the mean ± standard deviation (SD).

3. RESULTS AND DISCUSSION

3.1. Synthesis and characteristics of G3.0-Fu-IW and G3.0-Fu-Ado

One of the applications of nanomaterials in biomedical studies is their use for the enhancement of drug delivery and stability.[15] To achieve this purpose, drugs are entrapped in nanoparticles of different chemical nature. Dendrimers possessing internal cavities inside their structure are considered as perspective nanocarriers,[16] however, the toxicity strongly limits their application in the biomedicine. To overcome this problem in this study, PAMAM dendrimer G3.0 was coupled with fucoidan, a sulfated polysaccharide found in brown algae that ultilized as a supplement component in some functionally dietary products. It should be noted that due to basic nature of PAMAM dendrimer G3.0 its zeta potential is positive (39.3±1.87 mV). On the contrary the sulfated fucoidan has highly negative zeta potential of -90.63±1.14 mV. The electrostatic interaction between G3.0 and Fu is evident from the change in zeta potential, for G3.0-Fu it is -34.5±0.56 mV (table 1).

Both IW and Ado were encapsulated into dendrimer-fucoidan complex as described in section 2.2. The anticoagulant entrapment resulted in changes of zeta potentials. After IW and Ado encapsulation, the zeta potentials of resulting nanoparticles were shifted to less negative values (table 1). This shift might be explained by interaction of sulphate groups present in fucoidan and possessing strong negative charge with amino groups in IW or Ado. This interaction neutralizes the strong negative charge of the sulfate groups and shifts the zeta potential in the positive direction.

The drug entrapment efficiency was 95.63 % for G3.0-Fu-IW and 61.56 % for G3.0-Fu-Ado. The drug loading was 3.12 % and 2.98 % for G3.0-Fu-IWand G3.0-Fu-Ado, respectively. The particle shape and size of the nanomaterials obtained wereanalyzed by the methods of TEM and DLS; zeta potential was measured as well (table 1).

The TEM results showed that the shape of nanoparticles was changed after anticoagulant encapsulation from amorphous in unbound state (Figure 1a) to relatively uniform close to round when loaded with IW and Ado (figure 1b and c).

The nanoparticle size was also changed after encapsulation. The data of TEM showed the increase in the size from 32.35±5.09 nm for G3.0-Fu to 46.46 ±5.38 nm for G3.0-Fu-Ado and 72.76±10.93 nm for G3.0-Fu-IW (table 1), and this increase was proportional to the drug entrapment efficiency. The similar increase in nanoparticle size after encapsulation was observed by DLS (table 1). Although the size of nanoparticles determined in DLS experiments was larger than that observed by TEM, the size changes correlated with entrapment efficiency as well. The larger nanoparticle size observed by DLS might be explained by the hydrophilic nature of G3.0-Fu. In water, G3.0-Fu nanoparticles become highly solvated that results in apparent increase in their size.

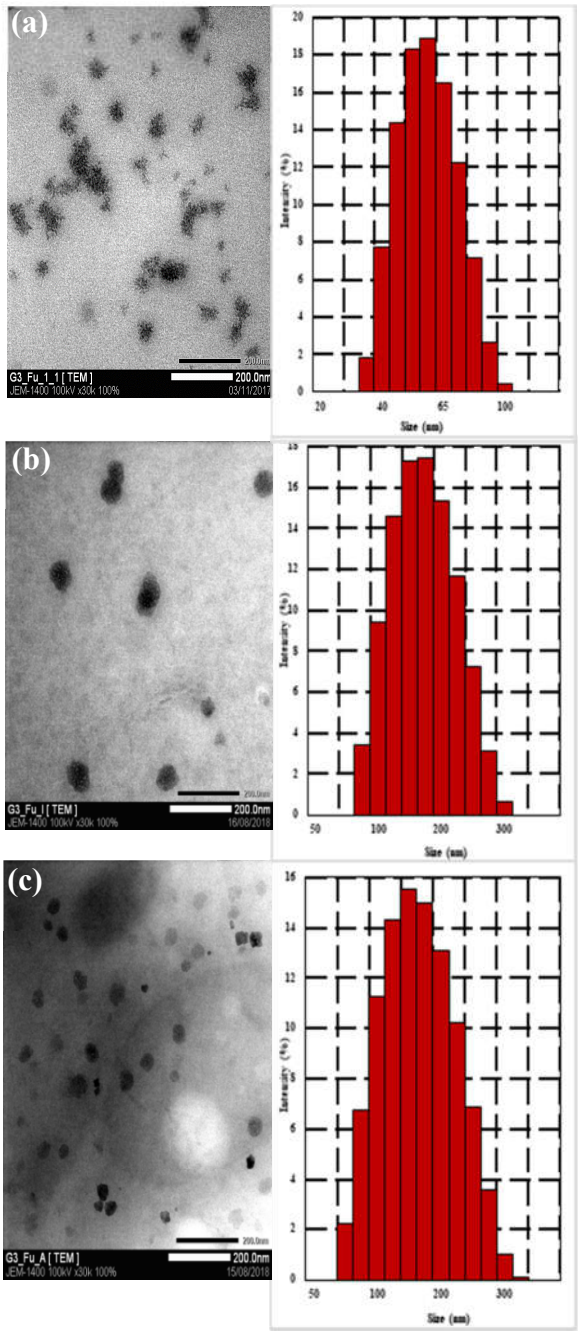

Figure 1: Morphology and size distribution of G3.0-Fu (a), G3.0-Fu-IW (b) and G3.0-Fu-Ado (c)

Table 1: The results of zeta, DLS and TEM measurement and anticoagulant content in G3.0-Fu nanomaterials

Sample	Zeta (mV)	Particle Size (nm)		Anticoagulant content (%)	
		DLS	TEM	EE	DL
G3.0-Fu	-34.5±0.56	70.23±2.61	32.35±5.09	-	-
G3.0-Fu-IW	-18.47±0.87	158.67±2.57	72.76±10.93	95.63	3.12
G3.0-Fu-Ado	-19.57±2.28	153.43±4.48	46.46±5.38	61.56	2.98

EE: drug entrapment efficiency; DL: drug loading.

Results of TEM, DLS and zeta potential measurements suggest that IW and Ado are entrapped by G3.0-Fu nanomaterials. After the loading with anticoagulants, the nanoparticles are more uniform and spherical in shape.

3.2. Synthesis and characteristics of Hep-P123-IW and Hep-P123-Ado

Recently novel amphiphilic heparin-pluronic P123 copolymer Hep-P123 with a great potential for drug delivery was developed.[14] IW and Ado were encapsulated in this copolymer as described in section 2.3. The zeta potential measurements showed the increase in the potential after anticoagulant incorporation in nanomaterial (table 2). Hep-P123 had the zeta potential of -60.52±1.73 mV which after IW and Ado encapsulation was changed to -38.03 ± 2.42 mV for Hep-P123-IW and -51.47 ± 0.64 mV for Hep-P123-Ado. This increase indicated that the interaction of IW and Ado with Hep-P123 resulted in shielding of strong negative charge of sulphate groups in heparin by interaction with amino groups of anticoagulants. Similarly to G3.0-Fu nanomaterials, the shape and size of the Hep-P123 nanomaterialswere analyzed by the methods of TEM and DLS (table 2).

Table 2: The results of zeta, DLS and TEM measurement and anticoagulant content in Hep-P123 nanomaterials

Sample	Zeta (mV)	Particle Size (nm)		Anticoagulant content (%)	
		DLS	TEM	EE	DL
Hep-P123	-60.52±1.73	145.23±6.42	74.26±9.97	-	-
Hep-P123-IW	-38.03±2.42	176.63±5.69	31.42±10.94	95.66	6.03
Hep-P123-Ado	-51.47±0.64	164.20±2.36	27.2±6.97	69.76	6.45

EE: drug entrapment efficiency; DL: drug loading.

The data of DLS demonstrated the increase in apparent nanoparticle size after IW and Ado encapsulation and this increase was correlated with drug loading efficiency (table 2). As with G3.0-Fu nanomaterials thedrug entrapmentefficiencies were fairly high and reached 95.66 % for Hep-P123-IW and 69.76 % for Hep-P123-Ado. In contrast to DLS data, the results of the TEM analysis showed the decrease in nanoparticle size after anticoagulant encapsulation from 74.26±9.97 nm for the Hep-P123 to 31.42±10.94 nm for Hep-P123-IW and to 27.2±6.97 nm for Hep-P123-Ado. This decrease inversely correlated with drug loading which was 6.03 % for Hep-P123-IW and 6.45 % for Hep-P123-Ado. The higher was drug loading, the smaller was the nanoparticle size (table 2, figure 2).

To explain this decrease, we should consider the possible interactions within nanomaterials. Due to the presence of strongly charged negative sulphate groups in heparin, Hep-P123 nanoparticles have a strong negative zeta potential (table 2).

However Hep-P123 is amphiphilic due grafted hydrophobic P123. In water, the hydrophobic parts of Hep-P123 tend to form compact structure; however the strong negative charges in heparin keep Hep-P123 in highly unfolded state. When the heparin negative charges are involved in interactions with amino groups of encapsulated IW and Ado, the hydrophobic interactions prevail and nanoparicles acquire more compact structure. This is what is observed for Hep-P123-IW and Hep-P123-Ado under TEM study where solvating water was removed before

measurements. This effect was not seen in DLS measurements carried out inaqueous solution. In water, the energy of hydrophobic interactions, probably, is not high enough to overcome the solvation energy even after shielding of strong negative charges by anticoagulants.

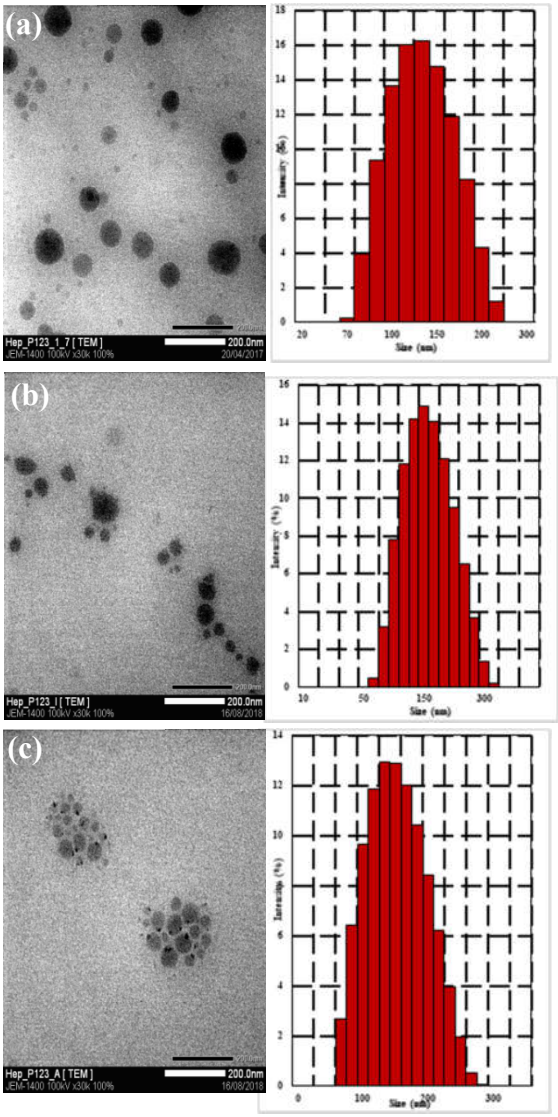

Figure 2: TEM images and size distribution (DLS) of Hep-P123 (a), Hep-P123-IW (b), Hep-P123-Ado (c)

Similarly to G3.0-Fu nanomaterials, the data of TEM, DLS and zeta potential measurements suggest that IW and Ado are encapsulated by Hep-P123 nanomaterials. The shapes of nanomaterials were not changed after the loading with anticoagulants and remained uniform and spherical.

4. CONCLUSION

In this work, we have synthesized two types of nanomaterials loaded with low molecular weight anticoagulants IW and Ado. One nanomaterial was complex of PAMAM dendrimer generation G3.0 and fucoidan, other one - amphiphilic heparin-pluronic P123 copolymer. The encapsulation of anticoagulants was confirmed by the changes in nanoparticle size and shape as well as by increase in zeta potential. The drug entrapment efficiencies were fairly high and ranged from 61.56 % for G3.0-Fu-Ado to 95.66 % for Hep-P123-IW. Drug loading was in the range from 2.98 % for G3.0-Fu-Ado to 6.45 % for Hep-P123-Ado, this parameter being higher for Hep-P123 nanomaterials.

This work lays the basis for further studies of nanomaterial encapsulated anticoagulant and shows the prospect for the application of G3.0-Fu and Hep-P123 drug-loaded platform in the biomedical field.

Acknowledgement. *The reported study was funded by RFBR according to the research project №18-54-54006 and by VAST according to the research project QTRU01.03/18-19.*

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