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FISH SCALE DERIVED COLLAGEN/GINSENOSIDE RB1 BIOCOMPOSITES: PREPARATION, CHARACTERIZATION AND THEIR HEMOSTATIC ABILITY[#]

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Abstract. This paper reports the characteristics and hemostatic ability of the collagen/ginsenoside Rb1 composites with different ginsenoside Rb1 content, in which, collagen was extracted from fresh water fish scales and ginsenoside Rb1 was extracted from *Panax notoginseng*. Glucose and glutaraldehyde were used as crosslinking agents for collagen molecules. Infrared (IR) spectroscopy and Field emission scanning electron microscopy (FESEM) methods were applied to assess the functional groups, interactions and morphology of the collagen/ginsenoside Rb1 composites. Glutaraldehyde exhibits a positive effect on improvement for dispersion of ginsenoside Rb1 in collagen matrix as well as on the interactions between ginsenoside Rb1 and collagen. In addition, the biocompatibility of the collagen/ginsenoside Rb1 in simulated body fluid was evaluated by ultraviolet-visible spectroscopy (UV-Vis) method. The obtained result shows that ginsenoside Rb1 can release well from the composite containing 1 wt.% of ginsenoside Rb1. Besides, the hemostatic ability of the composites was also tested and discussed.

Keywords: fish scale collagen, ginsenoside Rb1, characteristics, hemostatic ability, clotting time.

Classification numbers: 2.7.1, 2.9.4.

1. INTRODUCTION

Collagen is a natural polymer of non-toxic, biodegradability, biocompatibility and bioactivity, thus, it has been used in many fields such as pharmacy, agriculture, food, cosmetic,

[#]This paper is dedicated to the 40th anniversary of Institute for Tropical Technology if accepted for publication.

biomedical, etc. In biomedicine, collagen is a potential carrier for drug delivery [1 - 4], as a hemostatic agent [5 - 6] or as a burn treatment agent [7] and so on. In recent years, collagen derived from fish is known as a new biomaterial to replace for collagen sourced land animals thanks to great adsorption ability, good biocompatibility, no infectious disease, no hindrance to religion [8, 9]. Fish collagen has been applied for promotion of the growth of blood and lymphatic vessels [8], drug delivery [9, 10] and wound healing [11]. The application of fish collagen, especially collagen derived from fish scales, as hemostatic materials has been limited in research. Therefore, in this work, using collagen extracted from fresh water fish scales to make hemostatic agent has focused on study. However, one disadvantage of fish scale collagen is low denatured temperature, so, it is less stable and difficult to storage. One efficiency pathway to overcome this limitation is modification of fish scale collagen. Physical modification or chemical modification process can cause the change in bioactivity of collagen due to the hydrolysis or crosslinking of collagen chains [12]. The modification of collagen with crosslinking agents such as glutaraldehyde can enhance the adhesion ability of hemostatic agent with wounds [13].

For hemostatic agents, the treatment of wounds and promoting wound healing after bleeding is necessary. *Panax pseudoginseng* has effect on hemostatic, eliminating inflammatory pain, reducing blood pressure and cholesterol [14, 15]. Ginsenoside Rb1 - one of main compositions extracted from *Panax pseudoginseng* [16, 17] is able to improve blood circulation and treat sepsis [18]. Thus, for this research, ginsenoside Rb1 has been chosen to support fish scale collagen in hemostatic and to promote wound healing. Moreover, the formation of hydrogen bonding between hydroxyl groups in ginsenoside Rb1 and amide groups in collagen can be expected as for the formation of a synergistic effect in hemostatic ability of collagen and ginsenoside Rb1. The chemical structures of collagen and ginsenoside Rb1 were presented in Fig. 1.

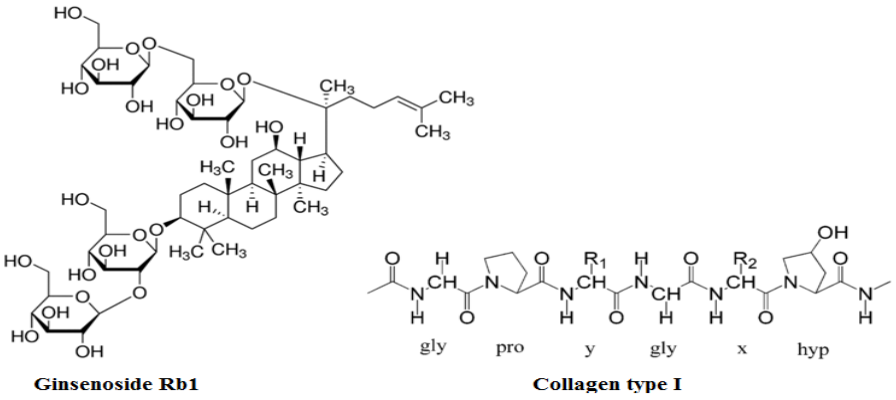

Figure 1. Chemical structures of ginsenoside Rb1 and collagen type I.

The purpose of this work is successful preparation of fish scale collagen/ginsenoside Rb1 composites with fish scale collagen modified with glucose or glutaraldehyde as crosslinking agents. The characteristics and *in-vitro* hemostatic ability of the composites were investigated and discussed.

2. MATERIALS AND METHOD

2.1. Materials

Collagen was extracted from fresh water fish scales which were collected at some Ha Noi markets by biochemical method using pepsin enzyme for hydrolysing collagen. Ginsenoside Rb1 (extracted from *Panax pseudoginseng*, in white powder form, 98 %) was provided by Institute of Medicinal Materials. Some other chemicals: glutaraldehyde, acetic acid 99.5 %, ethanol, glucose, HCl 37 %, KCl, NaCl, $NaHCO_3$, $CaCl_2$, Na_2HPO_4.2H2O, $MgCl_2.6H_2O$, $KH_2PO_4.7H_2O$, $MgSO_4.7H_2O$ were purchased from China.

2.2. Preparation of collagen/ginsenoside Rb1 composites

The preparation of the collagen/ginsenoside Rb1 composites as following: Firstly, collagen was dissolved in 30 mL of 1 % acetic acid solution on a magnetic stirrer. Next, 1 mL of glucose solution or 1 mL of glutaraldehyde solution was dropped slowly in collagen solution and the solution was stirred for 30 min to form crosslinked collagen solution. Ginsenoside Rb1 was dissolved in 10 mL of ethanol to form ginsenoside Rb1 solution. Then, ginsenoside Rb1 solution was dropped slowly into crosslinked collagen solution and the mixture was ultrasonicated at a speed of 20000 rpm for 30 min before magnetic stirring for 30 min to reach stable. Thereafter, the mixture was iced with ice + salt mixture. The precipitate was obtained by centrifuging at 6000 rpm for 5 min. After that, the solid part was natural evaporation at room temperature. The collagen/ginsenoside Rb1composites with different component ratio were designed in Table 1.

Table 1. Composition, abbreviation, shape and status of the collagen/ginsenoside Rb1 composites.

Abbreviation	Collagen (g)	Rb1 (g)	Glutaraldehyde (g)	Glucose (g)	Shape	Status
1RCo-Gluco	0.3	0.003	0	0.003	Film	Easily absorb moisture from the air
5RCo-Gluco	0.3	0.015	0	0.003	Powder	Normal storage
1RCo-Gluta	0.5	0.005	0.0025	0	Powder	Normal storage
5RCo-Gluta	0.5	0.025	0.0025	0	Powder	Normal storage

2.3. Characterization

Infrared (IR) spectra of the collagen/ginsenoside Rb1 composites were taken on a Nicolet iS10 spectrophotometer (Thermo Scientific, USA) in the wavenumbers from 400 to 4000 cm^{-1} with a resolution of 8 cm^{-1} and averaging scans of 32 times. Field emission scanning electron microscopy (FESEM) was performed using a FESEM S-4800 machine (Hitachi, Japan) at different magnification. Ultraviolet - visible (UV-Vis) spectra of the composites were recorded on a UV-Vis spectrophotometer (CINTRA 40, GBC, USA) in a wavelength from 200 to 800 nm.

2.4. *In-vitro* biocompatible study

Simulated body fluid (SBF) contains composition of ions similar to human blood. The composition of SBF listed in Table 2. We prepared the SBF solution from analytical chemicals.

The *in-vitro* ginsenoside Rb1 release test from the collagen/ginsenoside Rb1 composites was carried out following: 0.2 g of each sample was introduced in 200 mL of SBF, then, the

mixture was stirred continuously with a speed of 400 rpm at 37 ^{o}C. After time intervals, 5 mL of withdrawn solution was taken on the UV-Vis device to obtain the optical density value at the maximum of absorbance wavelength [19]. Then, the solution was poured into the mixture to maintain the total volume. The concentration of released ginsenoside Rb1 from the composites was calculated basing on optical density and the calibration equations of ginsenoside Rb1 in SBF ($y = 16183x + 0.0296$, $R^2 = 0.9972$, $\lambda_{max} = 208.42$ nm), in which x is the concentration of ginsenoside Rb1 (mol/L) and y is the optical density. The ginsenoside Rb1 release percentage can be determined by the following equation:

$$\text{Release [\%]} = m_{(t)}.100/m_{(0)} \quad (1)$$

where $m_{(0)}$ and $m_{(t)}$ represent the amount of ginsenoside Rb1 loaded and amount of ginsenoside Rb1 released at a time t, respectively.

Table 2. Composition of 1 L of SBF.

Chemicals	Concentration (g/L)
NaCl	8.00
$NaHCO_3$	0.35
KCl	0.40
$Na_2HPO_4.2H_2O$	0.48
$MgCl_2.6H_2O$	0.10
$CaCl_2$	0.18
$KH_2PO_4.7H_2O$	0.06
$MgSO_4.7H_2O$	0.10
Glucose	1.00

2.5. Assessment of hemostatic ability

Experiment to assess the ability to stop bleeding of the collagen/ginsenoside Rb1 composites was carried out at Military Medical University. 1 mL of whole blood is dripped directly from an intravenous line (taken at the front of the elbow) into a 5 mL tube set containing 3.5 mg of the composites or nothing (control group). No anticoagulants were used. After 1 mL of blood is put into the tube, it is covered and slowly reversed continuously until a blood clot forms. The number of seconds until clot formation is recorded for each tube. Staffs checking the formation of blood clots do not know the materials in the tubes [20].

3. RESULTS AND DISCUSSION

3.1. Status of the collagen/ginsenoside Rb1 composites

Table 1 summarizes the status of obtained collagen/ginsenoside Rb1 composites prepared at different conditions. It can be seen that the crosslinking agent and component ratio can affect on shape and status of the composites. The 5RCo-Gluco, 1RCo-Gluta, and 5RCo-Gluta samples

were formed in powder and can store normally while 1RCo-Gluco and 10RCo-Gluco samples were made in film and ease absorb moisture from the air, so, it is difficult to store them. Herein, glutaraldehyde can cross link with collagen chains more strongly than glucose, thus, fibril structure of collagen can be changed leading to the composites formed in powder. The ginsenoside Rb1 content also has effect on product shape of the composites. When using glucose as a crosslinking, 5 wt.% of ginsenoside Rb1 is suitable for preparation of the composites and sample storage at the normal conditions. As content of ginsenoside Rb1 smaller than 5 wt.%, glucose makes weaker crosslinking, thus, this content of ginsenoside Rb1 is not enough to support the change in structure of collagen, resulting in the composite is in film form. The moisture absorbance ability can limit application of the composites in reality.

3.2. Infrared spectra

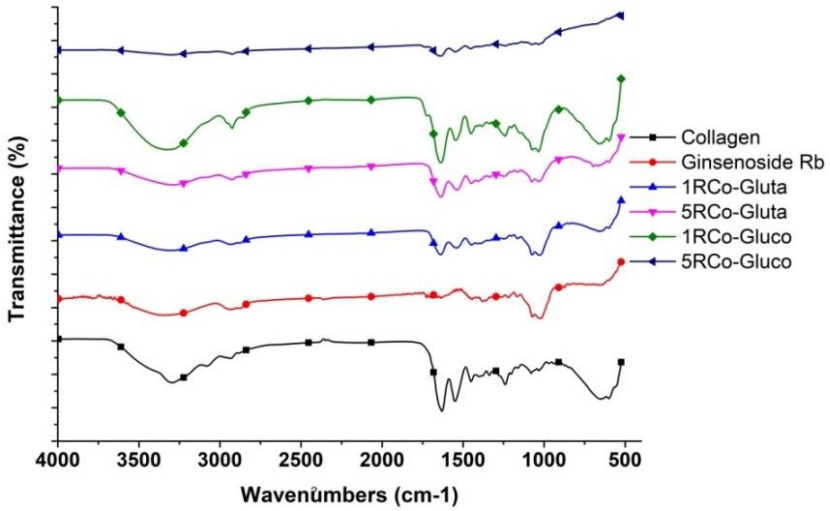

Figure 2. IR spectra of collagen, ginsenoside Rb1 and the collagen/ginsenoside Rb1 composites.

IR spectra of collagen, ginsenoside Rb1 and the collagen/ginsenoside Rb1 composites were shown in Fig. 2. Some characteristic peaks of ginsenoside Rb1 were placed at 3423, 2937, 1636, 1453 and 1384, 1268, and 1076 cm^{-1} corresponding to vibrations of O-H (stretching), C-H (stretching), O-H (bending) and C=C (stretching), C-H (scissoring), CH_2 (rocking), and C-C (stretching) groups, respectively in the IR spectrum of ginsenoside Rb1 [3]. The vibrations of amide A and amide B (3294 and 3076 cm^{-1}), CH groups (2934 cm^{-1}), amide I (C=O stretching vibration) (1630 cm^{-1}), amide II (amide N-H bending vibration) (1546 cm^{-1}) and amide III (C-N stretching vibration) (1238 cm^{-1}) groups were found in the IR spectrum of collagen [1, 3].

As compared with the IR spectra of collagen and ginsenoside Rb1, the adsorbance peaks characterized for functional groups of collagen and ginsenoside Rb1 were assigned on the IR spectrum of collagen/ginsenoside Rb1 composites. Table 3 lists the vibrations of some functional groups in the collagen, ginsenoside Rb1 and collagen/ginsenoside Rb1 composites. As can be seen that when combination of ginsenoside Rb1 and collagen, the position of amide vibration of collagen was slightly shifted due to interactions between hydroxyl groups in ginsenoside Rb1 and amide groups in collagen through hydrogen bonds. On the other hand, the crosslinking of glucose and glutaraldehyde with collagen also contributed shift the peaks characterized of amide groups on the IR spectra of the composites [21]. The NH_2 groups in

collagen linked to C=O groups in glutaraldehyde can lead to the decrease in intensity of peak characterized for amide II vibrations in IR spectra of collagen/ginsenoside Rb1 composites [22].

Table 3. Wavenumbers corresponding to vibrations characterized for some functional groups of collagen, ginsenoside Rb1 and the collagen/ginsenoside Rb1 composites.

Sample	Wavenumbers (cm^{-1})				
	Amide A, NH, OH	CH	Amide I, C=O, C=C	Amide II, NH, CH	Amide III, C-N, CH_2 (rock)
Collagen	3294	2934	1630	1546	1238
Ginsenoside Rb1	3423	2937	1636	1453	1268
1RCo-Gluco	3333	2924	1637	1547	1236
5RCo-Gluco	3305	2924	1636	1540	1238
1RCo-Gluta	3300	2936	1639	1539	1240
5RCo-Gluta	3289	2923	1635	1537	1240

3.3. Morphology

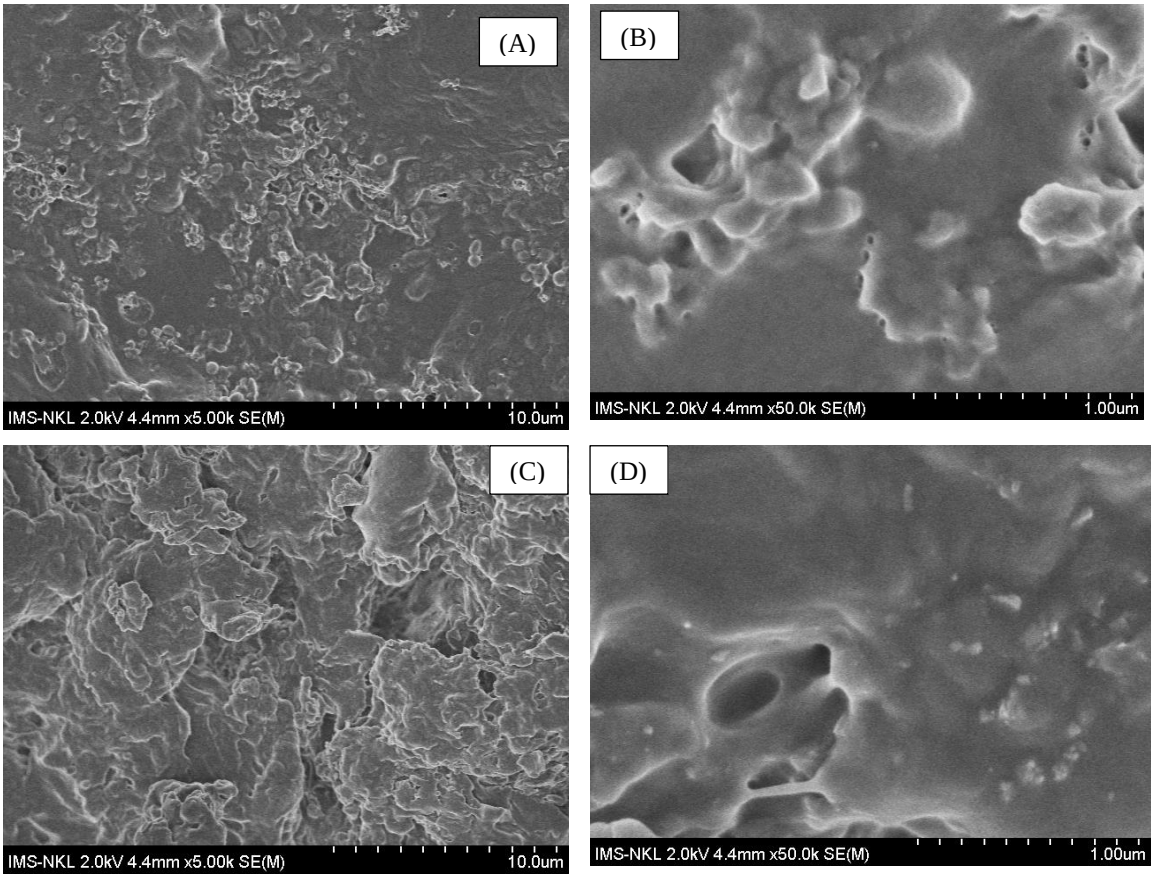

Figure 3. FESEM images of 5RCo-Gluco (A, B) and 5RCo-Gluta (C, D) composites at magnification of 5.000 times (left) and 50.000 times (right).

Due to the moisture absorbance of 1RCo-Gluco and 10RCo-Gluco film composites, the powder composites exhibit dominantly in real application. To consider the effect of crosslinking agents on morphology of the composites, FESEM images of 5RCo-Gluco and 5RCo-Gluta samples were taken at magnification of 5.000 and 50.000 times.

Compared FESEM images in Fig. 3, the 5RCo-Gluta sample has regular structure more than 5RCo-Gluco sample. At the magnification of 5.000 times, it cannot see the appearance of dispersed phase in the 5RCo-Gluta composite while ginsenoside Rb1 particles in size of 0.5-1 µm were appeared on the surface of the 5RCo-Gluco sample. The basic size of ginsenoside Rb1 particles in collagen matrix is about 100-200 nm but these particles agglomerated to each other in the 5RCo-Gluco sample (Fig. 3B). For the 5RCo-Gluta sample, the ginsenoside Rb1 was dispersed uniformly in collagen matrix with particle size of 50-100 nm (Fig. 3D). From this result, it can recognize that glutaraldehyde is more suitable for crosslinking collagen than glucose to obtain the composites having uniform structural morphology. The good dispersion of ginsenoside Rb1 in collagen matrix in the presence of glutaraldehyde crosslinking agent has been considered as an important key in control drug release as well as hemostatic ability of the composites.

3.4. Drug release study

Drug release study in simulated body fluids (SBF) plays an important role in evaluation of bioavailability of drug. In this study, the ginsenoside Rb1 release content from the 1RCo-Gluta and 5RCo-Gluta composites in SBF is tested and performed in Fig. 4.

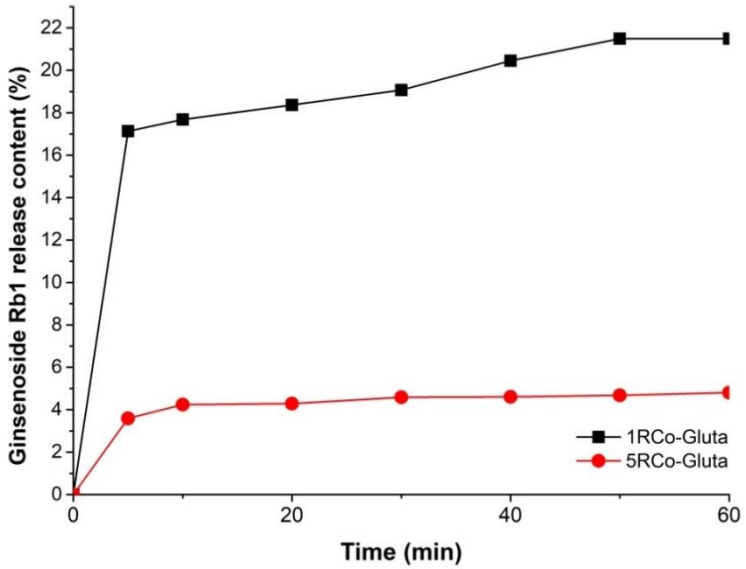

Figure 4. Ginsenoside Rb1 release content from collagen/ginsenoside Rb1 composites with different content of ginsenoside Rb1 in SBF.

The release process of ginsenoside Rb1 from the composites was divided into two stages: fast stage for 10 first minutes of testing and then, slow stage for following minutes and reached stable after 50 minutes of testing. The ginsenoside Rb1 was released slowly with low content from the 5RCo-Gluta sample, after 5 and 50 minutes of testing, the ginsenoside Rb1 release

content was reached 3.59 and 4.68 %, respectively. The ginsenoside Rb1 released from 1RCo-Gluta sample was much faster and larger than that from the 5RCo-Gluta sample. The ginsenoside Rb1 release content was 17.13 and 21.49 % after 5 and 50 minutes of testing, respectively. This can be explained by the interaction ability of ginsenoside Rb1 and collagen at low content better than that at high content of ginsenoside Rb1.

3.5. Hemostatic ability

Table 4 displays the clotting time of ginsenoside Rb1, collagen and collagen/ginsenoside Rb1 composites. As presented in Table 1, the 1RCo-Gluco sample is in film shape and easy to absorb moisture from the air. This can cause errors in clotting time testing; therefore, we didn't test the clotting time of this sample. It can be seen that clotting time of ginsenoside Rb1 was higher than that of control sample, meaning that ginsenoside Rb1 is not efficiency in promotion of blood clot process. The collagen and composites can promote the formation of blood clots, thus, their clotting time is lower than that of the control sample. The hemostatic mechanism of collagen materials is it helps to form the prothrombinase complex, which converts prothrombin into thrombin - a substance that plays a huge role in hemostasis. Fibrinogen under the action of thrombin will create a fibrin net that holds platelets and other components of the blood to create a stable blood clot that is capable of stopping bleeding [5, 6].

Among investigated samples, the 1RCo-Gluta composite had the highest clotting time, 134 seconds. This can due to the synergistic effect of collagen and ginsenoside Rb1 as well as better diffusion ability of ginsenoside Rb1 from the composite at low content into SBF as above discussed.

Table 4. Clotting time of ginsenoside Rb1, collagen and collagen/ginsenoside Rb1 composites.

Sample	Clotting time (second)
Control	495
Ginsenoside Rb1	551
Collagen	207
1RCo-Gluta	134
5RCo-Gluta	278
5RCo-Gluco	267

4. CONCLUSIONS

In this work, the influence of crosslinking agents on preparation and properties of the collagen/ginsenoside Rb1 composites has been investigated. The status of products indicated that some samples were obtained in powder and can be stored normally. The IR spectra of samples showed the existence of hydrogen bonding between collagen and ginsenoside Rb1 in the presence of crosslinking agents. The FESEM images expressed that ginsenoside Rb1 can disperse well in collagen matrix with 50 - 100 nm in size in the presence of glutaraldehyde. The ginsenoside Rb1 can release from the composites better when using 1 wt.% of ginsenoside Rb1. Clotting time result pointed 1RCo-Gluta composite has best hemostatic ability among tested samples. Combination of obtained results, the 1RCo-Gluta composite with composition ratio of

collagen: ginsenoside Rb1: glutaraldehyde = 200:2:1 can be applied as a potential hemostatic agent.

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Authorship contribution: Nguyen Thuy Chinh designed the study, processed data and wrote the paper. Nguyen Thuy Tien and Vu Quoc Manh performed experiment and data analysis. Thai Hoang revised the paper. All the authors have read and approved the final version of the manuscript.

Declaration of competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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