

Preparation of a novel composite nanofiber gel-encapsulated human placental extract through layer-by-layer self-assembly

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Received November 4, 2014; Accepted December 2, 2015

DOI: 10.3892/etm.2016.3084

Abstract. Aqueous human placenta extract (HPE) has been previously used to treat chronic soft tissue ulcer; however, the optimal dosage of HPE has yet to be elucidated. The present study investigated a novel nanofiber gel composed through layer-by-layer (LbL) self-assembly, in which HPE was encapsulated. IKVAV, RGD, RAD16 and FGL-PA were screened and combined to produce an optimal vehicle nanofiber gel through LbL assembly. Subsequently, the aqueous HPE was encapsulated into this nanofiber at the appropriate concentration, and the morphology, particle size, drug loading efficacy, encapsulation rate, release efficiency and structure validation were detected. The encapsulation efficiency of all three HPE samples was >90%, the nanofiber gel exhibited a slow releasing profile, and the structure of HPE encapsulated in the nanofiber gel was unvaried. In conclusion, this type of novel composite nanocapsules may offer a promising delivery system for HPE.

Introduction

The wound healing potency of an aqueous human placental extract (HPE) has been clinically established in previous studies (1,2). HPE has numerous applications in the treatment of chronic soft tissue ulcers and in the stimulation of endogenous growth factors, while it possesses anti-inflammatory and antimicrobial properties (1,2). HPE efficiently activates adenosine A_{2A} receptors and is not degraded *in vivo*, meaning it is more efficient than other types of therapy, including vacuum sealing drainage, local application of growth factor, and traditional Chinese medicine (3). However, the use of

appropriate vehicles to maintain the biological activity of HPE during the delivery process is crucial to the success of the treatment (4,5).

Short poly-N-acetyl glucosamine (sNAG) nanofibers have been found to activate the integrin receptor on the surface of platelets, promote the formation of fibrous protein complexes and cell proliferation and migration, and stimulate the release of a variety of growth factors (6,7). The activated integrin receptor was also found to increase the expression of vascular endothelial growth factor (VEGF) and accelerate the formation of new blood vessels through a transcriptional factor-dependent signaling pathway (8). Furthermore, sNAG nanofibers may also accelerate the expression of α -defensins and defensin β -1 in endothelial and keratin cells (9). Therefore, self-assembled sNAG nanofiber gel encapsulating aqueous HPE can serve as an ideal matrix material and tool for the treatment of chronic soft tissue ulcers (9,10).

Layer-by-layer (LbL) self-assembly is a widely used technique of alternating the adsorption of materials onto a surface using complementary interactions, one layer of material at a time, in order to create nanometer-thin films (11,12). Due to its versatility and simplicity, the LbL self-assembly technique has been under intensive investigation for a wide range of purposes, including bone tissue engineering for vaccine delivery and the creation of biological interfaces (13,14). The development of stimuli-responsive LbL-assembled nanoparticles has advanced in recent years (15).

In the present study, the aqueous HPE was encapsulated in a novel nanocomposite gel fiber (16,17) prepared by the ideal formulation of IKVAV, RGD, RAD16 and FGL-PA through LbL self-assembly (17,18,19,20), and the morphology, particle size, drug loading, drug release efficacy, encapsulation rate and structure validation were examined. IKVAV is an amphiphilic molecule, also known as isoleucine-lysine-valine-alanine-valine. The sequence of IKVAV is C₁₆H₃₁O-NH-AAA GGG E IKVAV-COOH, and this was prepared by the solid phase synthesis method (21). RAD16 is a self-assembled peptide composed of alternating negative and positive amino acids (ACN-RADARADARADARADA-CONH₂). In addition, the FGL-PA sequence was C₂₂H₄₃O-NH-AAAGGGEVY VVAENQQGKSKA-COOH, and the RGD sequence was C₁₆H₃₁O-NHAAAAGGGS(PO₄)₃-RGD-COOH. The study aimed to investigate whether this novel type of composite nanocapsules may offer a promising delivery system for HPE.

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Key words: novel layer-by-layer self-assembly, nanocomposite gel fiber, aqueous extract of human placenta

Materials and methods

Materials. IKVAV (relative molecular mass of 1,351.6 with 98% purity; batch number, 20140520; Shanghai Bootech Bioscience & Technology Co., Ltd., Shanghai, China), RAD16 (relative molecular mass of 1207.34 with 98% purity; Shanghai Bootech Bioscience & Technology Co., Ltd.), FGL-PA (relative molecular mass of 2,485.92 with 98% purity; Shanghai Bootech Bioscience & Technology Co., Ltd.), and RGD (relative molecular mass of 1,207.34 with 99% purity; Shanghai Bootech Bioscience & Technology Co., Ltd.) were used in the present study.

Preparation of vehicle nanofiber gel. For the preparation of the vehicle nanofiber gel, 10 mg IKVAV, 10 mg RAD16, 10 mg FGL-PA and 10 mg RGD were added to a mixture of 0.1 M NaOH (400 μ l) and twice-distilled water (400 μ l). The mixture was then placed in 37°C for 30 min and stirred vigorously until a clear liquid was obtained. The pH value was detected using a pH meter (pH 9.4) and 0.1 M HCl was used to adjust the pH to 8.5–9.0. Distilled water was used to adjust the peptide concentration to 1 mg/ml. Next, 0.1 ml Dulbecco's modified Eagle's medium/nutrient mixture F12 (DMEM-F12; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was added to 0.1 ml peptide to trigger self-assembly.

A greater nanofiber gel viscosity resulted in better performance of the gel. Based on the preliminary results, IKVAV, FGL-PA and RGD were used for the formula screening (22). The appropriate proportion and concentration of IKVAV, FGL-PA and RGD were screened to prepare the optimum nanofibers gel for the appropriate times as follows: i) IKVAV:RGD = 2:1 (4.5 sec); ii) IKVAV:RGD = 1:1 (4 sec); iii) IKVAV:RGD = 1:2 (5 sec); iv) IKVAV:FGL-PA = 2:1 (5.5 sec); v) IKVAV:FGL-PA = 1:1 (6 sec); vi) IKVAV:FGL-PA = 1:2 (7 sec); vii) RGD:FGL-PA = 2:1 (8 sec); viii) RGD:FGL-PA = 1:1 (8.2 sec); ix) RGD:FGL-PA = 1:2 (8.5 sec); x) IKVAV:RGD:FGL-PA = 1:1:1 (11 sec); xi) IKVAV:RGD:FGL-PA = 3:2:1 (9 sec); xii) IKVAV:RGD:FGL-PA = 1:2:3 (10 sec).

Preparation of nanofiber gel-encapsulated HPE. HPE was obtained from a 28 year-old patient recruited from the Union Hospital of the Tongji Medical College (Wuhan, China) on February 2014. According to the appropriate proportion and concentration, IKVAV, FGL-PA and RGD were added into the HPE, and then mixed with an equal volume DMEM-F12 to trigger self-assembly at room temperature. The solution was then diluted 0, 50 and 100 times with distilled water and observed at magnifications of x30,000, x39,000, x65,000, x93,000 and x135,000 using a Tecnai-10 transmission electron microscope (Philips, Amsterdam, Netherlands).

Transmission electron microscopy. The morphology of the HPE nanofiber gels was analyzed using a Tecnai-10 transmission electron microscope (Philips). Briefly, following dilution (1:100) in water, the sample was negatively-stained with 1% (weight/volume) phosphotungstic acid (Shanghai Muhong Industrial Co., Ltd., Shanghai, China) for 5 min at 25°C. Subsequently, the sample was placed on copper film grids

(Wuhan Boster Biological Technology, Ltd., Wuhan, China) and observed using transmission electron microscopy after drying for 10 min at 25°C (23).

Particle size determination. After diluting 100-fold with distilled water, the mean particle size of the HPE nanofiber gel was determined using Zetasizer 3000 HS (Malvern Instruments Ltd., Malvern, UK). Three parallel measurements were performed for each sample as previously described (24).

Encapsulation efficiency and drug loading testing of HPE nanofiber gel. The encapsulation efficiency and drug loading was analyzed by dissolving ~5 mg HPE nanofiber gel in either 1.2 ml distilled water or absolute ethyl alcohol. Next, the mixture was centrifuged at 8,000 \times g for 10 min and the precipitation was resuspended in 1.2 ml distilled water. The supernatant and precipitation were detected using an ultra-violet (UV) detector (Alpha 1500; Shanghai Lab-Spectrum Instruments Co., Ltd., Shanghai, China) at 280 nm. The formula used to measure the encapsulation efficiency was as follows: Encapsulation efficiency = (concentration of supernatant + concentration of precipitation in demulsified samples - concentration of precipitation in non-demulsified samples)/total concentration of demulsified samples. The formula used to measure drug loading was as follows: Drug loading = actual drug concentration / (drug-loading particle + drug concentration). Three parallel measurements were performed for each sample.

Slow-releasing potential detection of HPE nanofiber gel. The slow-releasing potential of the HPE nanofiber gel was evaluated using a dialysis method. Briefly, dialysis bags (molecular weight cut-off, 1,000 Da) containing 0.1 ml HPE nanofiber gel were immersed in a thermostatic gas bath containing 20 ml release medium (phosphate-buffered saline, pH 7.2) at 37°C. At predefined intervals (0.25, 0.5, 1, 1.5, 2, 4, 6, 9, 12, 24, 48, 72, and 96 h), 1 ml aliquots of phosphate-buffered saline (Wuhan Boster Biological Technology, Ltd., Wuhan, China) were withdrawn and replaced with the same volume of release medium. The HPE release behavior of the nanofiber gel was determined using a UV spectrophotometer (Agilent 8453; Agilent Technologies, Santa Barbara, CA, USA) at 280 nm. The measured concentrations were calculated according to the standard curve, followed by calculation of the cumulative release rate (22).

Crystal structure validation of HPE nanofiber gel. The HPE stock solution and nanofiber gel were diluted 100 times with distilled water. The solution was stirred for 10 min at 20 \times g. The crystal structure of the HPE stock solution and HPE nanofiber gel was then evaluated using an X-ray diffractometer (Siemens D5000; Siemens AG, Munich, Germany). The condition of the diffraction was as follows: λ = 1.5064 Å; voltage, 20 kV; electric current, 20 mA; 2 θ scanning in the range of 10° to 80°; scanning frequency, 0.02° Q/S.

Statistical analysis. Statistical analysis was performed using the SPSS version 16 software (SPSS, Inc., Chicago, IL, USA). The statistical significance of differences was determined

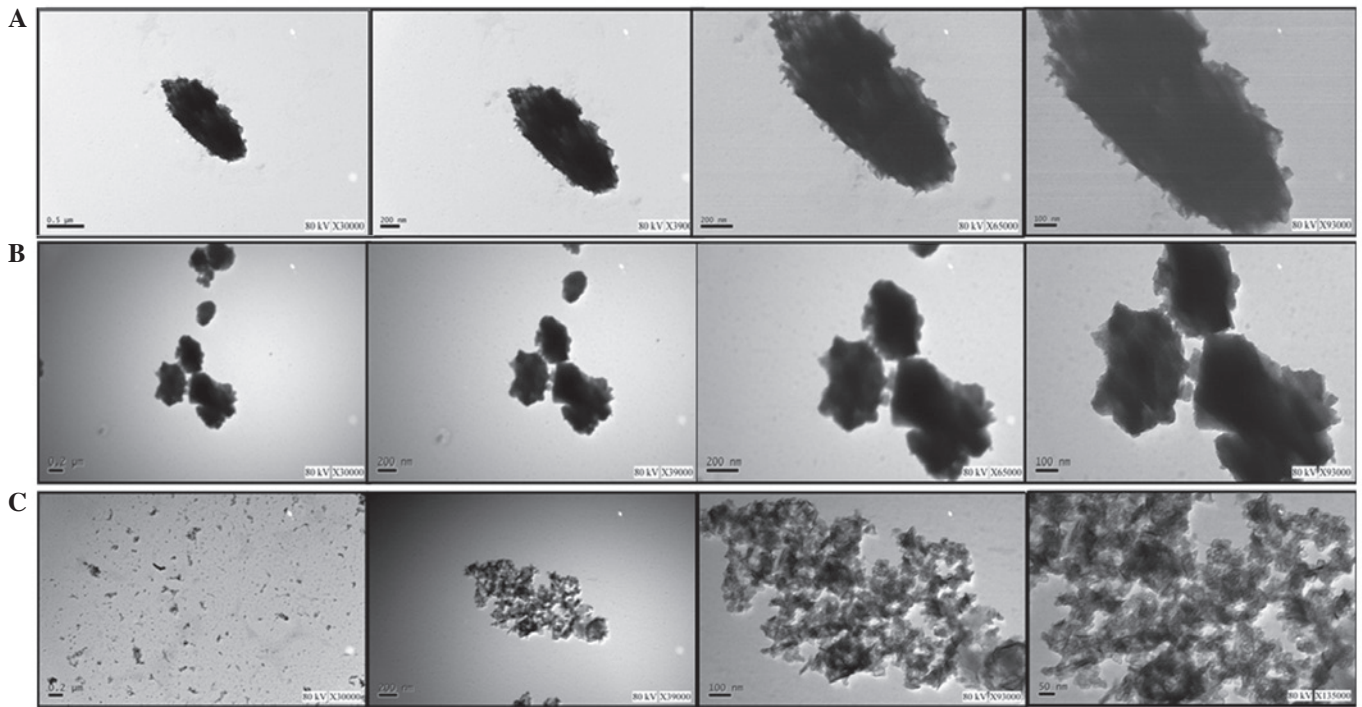


Figure 1. Morphology of human placental extract encapsulated in nanofibers, as observed using transmission electron microscopy. Different dilutions were used, including dilution by (A) 0, (B) 50 and (C) 100 times. The four images in each part of the figure were captured at different magnifications. In (A) and (B), the magnification is x30,000, x39,000, x65,000 and x93,000 (from left to right), while in (C) the magnifications are x30,000, x39,000, x93,000 and x135,000 (from left to right).

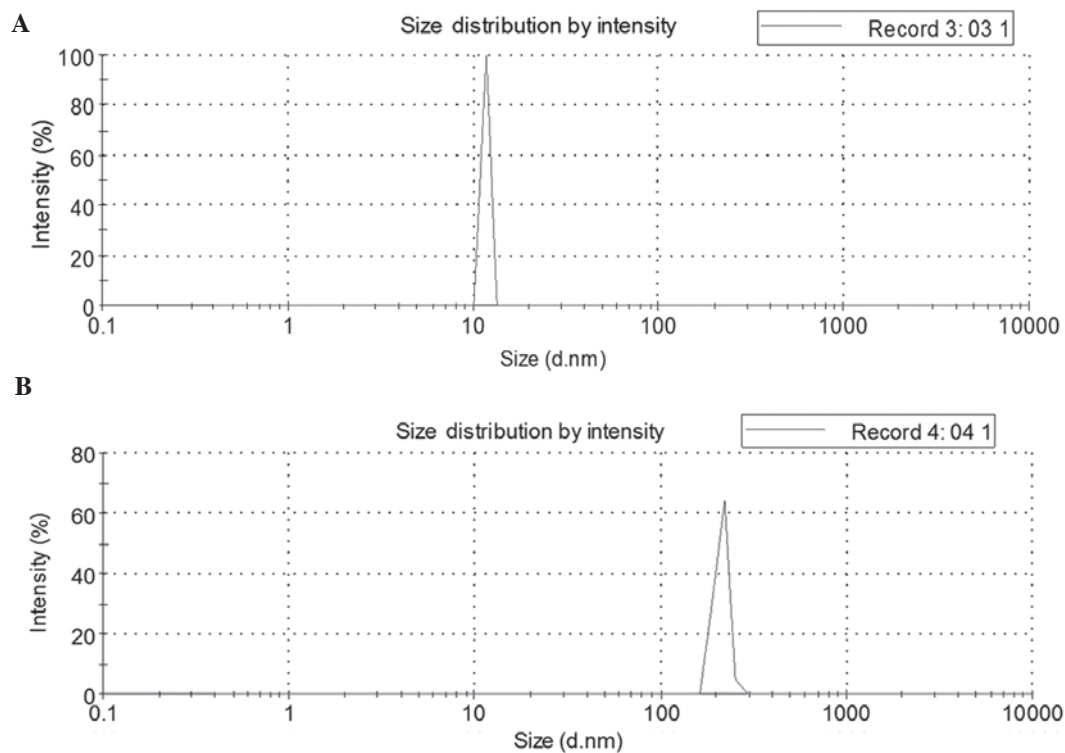


Figure 2. Mean particle size of (A) vehicle nanofiber gel and (B) human placental extract nanofiber gel.

using one-way analysis of variance. All statistical tests were two-sided, and $P < 0.05$ was considered to indicate a statistically significant difference. Results are presented as the mean \pm standard deviation.

Results

Preparation and characterization of HPE encapsulated in nanofiber gels. The IKVAV, RAD16, FGL-PA and RGD

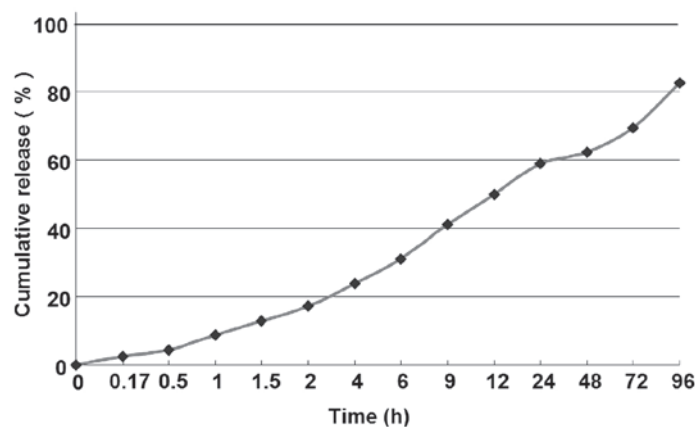


Figure 3. *In vitro* release profiles of the HPE encapsulated in nanofiber gels in phosphate-buffered saline (pH 7.2) at 37°C.

peptides were synthesized and used to form nanofiber skeletons using the self-assembly method via electrostatic and hydrophobic interactions. After a preliminary screening, IKVAV, RGD and FGL-PA were able to form nanofiber skeletons with greater viscosity. Twelve different combinations of IKVAV, RGD and FGL-PA were used for the formula screening. The results indicated that the optimal proportion of the nanofiber gel was IKVAV : RGD : FGL-PA = 1:1:1 and the optimal concentration was 0.6 mg/ml, as determined by observing the liquidity, diffusion time and viscosity.

Subsequently, the HPE was encapsulated into the nanofibers. The morphology of the HPE nanofibers was observed using transmission electron microscopy. The results revealed that the HPE nanofibers had a fibrous appearance under different dilutions. With the increase of the dilution, the nanofiber gel appeared to be more homogeneously dispersed. At a dilution of 100, the nanofiber gel was found to be directly observed (Fig. 1).

As shown in Fig. 2, the mean particle widths of the vehicle and of the HPE nanofiber gels were ~11.7 and 212 nm, respectively.

Encapsulation efficiency and *in vitro* release of HPE. The results of UV spectrophotometric analysis revealed that the encapsulation efficiency of all three HPE samples (dilution performed in triplicate) was >90% (96.8, 98.3 and 94.8%), with a mean encapsulation efficiency of $96.6 \pm 1.75\%$. The drug loading of the three samples (dilution performed in triplicate) was found to be 5.2, 5.13 and 5.11 mg/g, with a mean drug loading of 5.15 ± 0.04 mg/g (data not shown).

The *in vitro* release of HPE nanofiber gel was examined using a dialysis method. The drug release profile was performed under simulated physiologic conditions (pH 7.2). As shown in Fig. 3, the HPE nanofiber gel showed a sustained drug release profile, with a total HPE release from the nanofibers of 2.35–82.58%. The nanofiber gel exhibited a slow-releasing profile (>4 days).

Crystal structure validation of HPE nanofiber gel. The results indicated that the peak number and time of HPE nanofiber gel was similar to that of the HPE stock solution. The preliminary results confirmed that the structure of HPE encapsulated in the nanofiber gel was unvaried compared with the HPE structure.

Discussion

The absolute or relative reduction of exogenous growth factor and its receptor is one of the main reasons why the treatment of chronic soft tissue ulcer is challenging (25,26). The topical application of active growth factors can promote the healing of chronic ulcer (27); however, our previous study revealed that the exogenous growth factors were easily degraded and diluted due to their short half-life (28). Signaling pathways that are able to awaken the body's self-healing mechanisms are therefore required to promote the sustained release of endogenous growth factors. Recent studies have shown that adenosine A2A receptor activation may play an important role in the protection and promotion of wound healing in ischemia, hypoxia, inflammation, trauma and numerous other pathological processes (29,30). The adenosine A2A receptor activation can inhibit the generation of reactive oxygen species and promote the release of cytokines, such as tumor necrosis factor- α , accelerate osteoblast, fibroblast and fat precursor cell proliferation, as well as the release of VEGF, angiogenin and glutamine transferase II, and thus promoting wound healing (31,32). Therefore, selective activation of adenosine A2A receptors awakens the body's self-healing mechanisms (33).

Numerous studies have demonstrated the biological actions of HPE in various diseases, and HPE has been used in wound healing in several countries for years as a folk remedy (34,35). Another study suggested that HPE exerts an anti-inflammatory function that suppresses chemical mediators, and that these effects may be associated with innate immune functions (36). Furthermore, previous studies indicated that oligomeric DNA nucleotides, such as polydeoxyribonucleotide (PDRN), are linear DNA nucleotide polymers from HPE with a length range of 50–2,000 bp (37,38). PDRN can efficiently activate adenosine A2A receptors, without being degraded into small fragments, thus preventing the activation of other adenosine receptors (39,40). PDRN treatment for chronic soft tissue ulcer has been found to significantly increase wound granulation, thus promoting wound healing (41). However, the method used to promote the PDRN late release will directly affect its therapeutic effect on the chronic soft tissue ulcer.

To date, limited information is available regarding the dosage of HPE required for wound healing. In the present study, aqueous HPE was encapsulated in a novel nanofiber

gel prepared by the appropriate formulation of IKVAV, RGD, RAD16 and FGL-PA through LbL assembly. Subsequently, the morphology, particle size, drug loading efficacy, encapsulation rate, release efficacy and structure validation were examined. The results showed that the particle size of the HPE nanofiber gel was 212 nm, which was included in the 1-1,000 nanometer materials. Following the detection of the encapsulation efficiency and drug loading, the HPE nanofiber gel was found to have high drug loading (5.15 mg/g) and high encapsulation efficiency (96.6%). Furthermore, the HPE nanofiber gel exhibited a slow-releasing profile (>4 days). Investigation of the crystal diffraction structure confirmed that the structure of HPE that was encapsulated in the nanofiber gel did not undergo any apparent changes. Therefore, these novel composite nanocapsules may offer a promising delivery system for HPE.

Acknowledgements

The present study was supported by the National Natural Science Fund of China (grant no. 81271968).

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