ChinaRxiv 中X

AI translation · View original & related papers at
chinarxiv.org/items/chinaxiv-201806.00139

Fabrication and Biological Activity Evaluation of Hepatocyte Growth Factor-Loaded Poly(lactic-co-glycolic acid) Nanoparticles Postprint

Authors: Xian Wenjiao, Wang Xue' er, Zhang Lin

Date: 2018-06-15T00:00:00+00:00

Abstract

Objective: To investigate the optimal conditions for preparing poly(lactic-co-glycolic acid) (PLGA) nanoparticles, construct hepatocyte growth factor (HGF) nanoparticles, and evaluate their encapsulation efficiency, drug loading, recovery rate, release profile, and biological activity. Methods: Bovine serum albumin (BSA) PLGA nanoparticles were prepared using the double emulsion solvent evaporation method. Through orthogonal experimental design, with smaller particle size and higher encapsulation efficiency, drug loading, and recovery rate as evaluation indices, the preparation conditions of nanoparticles were optimized; HGF nanoparticles were prepared under the optimized conditions, and BCA kit and HGF-ELISA kit were respectively used to detect the encapsulation efficiency, drug loading, and release profile of BSA nanoparticles and HGF nanoparticles, while the biological activity of HGF nanoparticles was evaluated through CCK8 proliferation assay. Results: HGF nanoparticles prepared under optimized conditions showed uniform size with a particle size of 234.4±4.8 nm, encapsulation efficiency of (77.75±3.04)%, recovery rate of (49.33±9.34)%; the in vitro release profile exhibited an initial burst release followed by sustained release; HGF nanoparticles could promote the proliferation of keratinocytes. Conclusion: HGF nanoparticles prepared by the double emulsion solvent evaporation method under optimized conditions exhibited high encapsulation efficiency, good sustained-release effect, and biological activity.

Full Text

Preamble

Xian Wenjiao, Wang Xue' er, Zhang Lin
Department of Histology and Embryology, School of Basic Medical Sciences, Southern Medical University, Guangzhou 510515, China

Abstract

Objective: To explore the optimum conditions for preparing poly(lactic-co-glycolic acid) (PLGA) nanoparticles and evaluate the bioactivity of hepatocyte growth factor (HGF)-loaded PLGA nanoparticles. **Methods:** Bovine serum albumin (BSA)-loaded PLGA nanoparticles were prepared using a double emulsion-solvent evaporation method. The preparation process was optimized by orthogonal test with particle size, encapsulation efficiency (EE), drug loading (DL), and recovery rate as evaluation indexes. HGF-loaded nanoparticles were then prepared under the optimized conditions. The EE, DL, and release characteristics of both BSA-loaded and HGF-loaded nanoparticles were evaluated using a BCA kit and HGF ELISA kit. The bioactivity of HGF-loaded nanoparticles was assessed using CCK-8 proliferation assay. **Results:** The HGF-loaded nanoparticles prepared under optimized conditions exhibited uniform size with a mean diameter of 234.4±4.8 nm, an EE of (77.75±3.04)%, and a recovery rate of (49.33±9.34)%. The in vitro release profile showed an initial burst release followed by sustained release. HGF-loaded nanoparticles significantly promoted the proliferation of HaCaT keratinocytes in vitro. **Conclusion:** HGF-loaded nanoparticles prepared using the double emulsion-solvent evaporation method under optimized conditions possess high encapsulation efficiency, favorable sustained release properties, and good bioactivity.

Keywords: poly(lactic-co-glycolic acid) nanoparticles; orthogonal test; hepatocyte growth factor; sustained release

Introduction

Growth factors play crucial roles throughout all stages of skin wound healing. Animal experiments and clinical studies have demonstrated that various growth factors can promote wound healing. Hepatocyte growth factor (HGF) is a multifunctional growth factor that has been shown to accelerate wound healing. However, HGF exhibits poor stability in vitro and is readily degraded by enzymes in vivo, making it difficult to achieve persistent and effective local action. Additionally, rapid systemic diffusion of HGF can lead to various toxic side effects. Therefore, constructing a safe and effective sustained-release drug delivery system for HGF is essential for its clinical application. Poly(lactic-co-glycolic acid) (PLGA) has been widely used in pharmaceutical applications. PLGA nanoparticles can achieve high encapsulation efficiency for growth factors, enhance their stability and bioavailability, and the sustained release of drugs combined with PLGA' s metabolic product lactate can collectively promote wound healing. Nevertheless, few studies have reported on sustained-release drug delivery systems for HGF. In this study, we employed PLGA as the encapsulation material, utilized orthogonal experimental design to optimize the preparation conditions for PLGA nanoparticles, and prepared HGF-loaded nanoparticles under these optimized conditions for subsequent quality evaluation and biological activity assessment.

Materials and Methods

1.1 Reagents and Instruments

PLGA (50:50, molecular weight 24,000-38,000), polyvinyl alcohol (PVA, molecular weight 13,000-23,000) were purchased from Sigma. Mannitol and sucrose were obtained from Guangzhou Chemical Reagent Factory. Equipment included an ultracentrifuge (Beckman), freeze dryer (CHRIST), Malvern nanosizer (UK), transmission electron microscope (Japan), BCA kit (Beyotime), HGF (R&D), HGF ELISA kit (Dongge Biotech), and microplate reader Epoch (BioTek).

1.2 Preparation of PLGA Nanoparticles

Nanoparticles were prepared following a reported method with slight modifications. Briefly, an appropriate amount of BSA was dissolved in sterile water containing 10% mannitol, 5% sucrose, and 5% PEG400 as the aqueous phase. A predetermined amount of PLGA was dissolved in a mixture of dichloromethane and acetone (3:1) as the oil phase. The aqueous phase was added to the oil phase and sonicated for 1 min to form a primary emulsion. PVA solution was then added to the primary emulsion and sonicated for 1 min to form a double emulsion, which was dispersed in water and gently stirred with magnetic stirring for 4 h to evaporate the organic solvent. The mixture was centrifuged at 14,000 r/min for 20 min, and both supernatant and precipitate were collected. The supernatant was used to detect encapsulation efficiency, while the precipitate was washed twice with sterile water, freeze-dried for 24 h, and stored at 4°C for future use. For HGF-loaded nanoparticles, an appropriate amount of HGF was added to the aqueous phase.

1.3 Orthogonal Experimental Design for Optimization

Following orthogonal experimental design principles, an L9(3^4) orthogonal array was adopted to investigate three factors: PVA concentration, PLGA dosage, and ultrasonic power. The fourth column was left empty. Each factor had three levels, resulting in nine experimental runs. The specific design is shown in Table 1 .

1.4 Characterization of Nanoparticles

1.4.1 Measurement of Average Particle Size, Size Distribution, and Zeta Potential A certain amount of nanoparticles was dispersed in sterile water and diluted to a visibly turbid concentration. The suspension was then analyzed using a Malvern nanosizer.

1.4.2 Observation of Surface Morphology and Internal Structure A drop of sample was placed on a carbon-coated copper grid. Excess liquid was removed from the edge of the droplet with filter paper. The sample was negatively stained with 3% phosphotungstic acid aqueous solution for 2 min, after which

the stain was removed with filter paper. After drying at room temperature, the sample was observed under transmission electron microscopy.

1.5 Determination of Encapsulation Parameters

1.5.1 Encapsulation Efficiency Measurement The supernatant collected during centrifugation was analyzed. BSA-loaded nanoparticle concentration was measured using a BCA kit, while HGF-loaded nanoparticle concentration was determined using an HGF ELISA kit. Encapsulation efficiency was calculated as: (Amount of encapsulated protein drug / Total amount of encapsulated and unencapsulated protein drug) × 100%.

1.5.2 Drug Loading Measurement A predetermined amount of nanoparticles was weighed and dissolved in 0.5 mL dichloromethane. Then 0.5 mL sterile water was added to extract the protein from the nanoparticles. After centrifugation, the supernatant was collected. This extraction was repeated three times, and the supernatants were pooled. Concentrations were measured using BCA kit for BSA nanoparticles and ELISA kit for HGF nanoparticles. Drug loading was calculated as: (Amount of protein drug in nanoparticles / Total weight of nanoparticles) × 100%.

1.5.3 Recovery Rate Measurement All dried nanoparticles were collected and weighed on an analytical balance. The recovery rate was calculated as: (Total weight of obtained nanoparticles / Total weight of all materials input) × 100%.

1.5.4 BCA Kit Detection Procedure Protein standard solutions of predetermined concentrations were prepared. BCA working solution was prepared according to the manufacturer' s instructions and mixed thoroughly. Gradient volumes of standard solution and samples were added to corresponding wells of a 96-well plate (n=3) and incubated for 30 min. Working solution was then added, and the plate was incubated at 37°C. Absorbance was measured at 562 nm using a microplate reader. A standard curve was constructed to calculate sample concentrations.

1.5.5 HGF ELISA Kit Detection Procedure Standards and samples were added to corresponding wells of an ELISA plate. Blank wells were set up, and the plate was sealed and incubated at 37°C for 30 min. The plate was washed five times with wash buffer and dried by patting after each wash. Enzyme-labeled reagent was added to all wells except blank wells. The plate was sealed and incubated at 37°C for 30 min, then washed five times and dried. Substrate solutions A and B were added for color development, and the plate was sealed and incubated at 37°C for 15 min. Stop solution was added, and absorbance was measured at 450 nm using a microplate reader. A standard curve was constructed to determine sample concentrations.

1.6 In Vitro Release Study

A predetermined amount of nanoparticles was placed in a dialysis membrane with 5 mL PBS (pH 7.4). Release was performed at 37°C with shaking at 72 r/min. At time points of 6, 12, 24, 48, 72, 120, and 168 h, 500 L samples were withdrawn and replaced with an equal volume of fresh PBS. Samples were stored at 4°C until analysis. BSA nanoparticles were analyzed using BCA kit, while HGF nanoparticles were analyzed using ELISA kit. Cumulative release was calculated and release curves were plotted.

1.7 HaCaT Cell Culture

Human immortalized keratinocytes (HaCaT) were purchased from the Shanghai Institute of Chinese Academy of Sciences. Cells were revived under sterile conditions and seeded in 10 cm^2 culture dishes. They were cultured at 37°C in a 5% CO incubator until reaching 80-95% confluence, then digested with trypsin and passaged.

1.8 Cell Proliferation Assay

The experiment was divided into four groups: control, BSA nanoparticle, HGF solution, and HGF nanoparticle groups. Cell suspension was seeded in 96-well plates at a density of 3.0×10^3 cells per well in high-glucose medium containing 10% FBS and cultured at 37°C in a 5% CO incubator. After overnight incubation, the medium was replaced with 0.1% FBS medium for starvation for 12 h, followed by addition of respective stimuli according to the experimental protocol. Cells were then cultured in maintenance medium. At 12, 24, 48, and 72 h time points, 10 L CCK-8 reagent was added to each well and incubated for 3 h. Absorbance was measured at 450 nm to assess cell proliferation.

1.9 Statistical Analysis

Data are expressed as mean ± standard deviation. Statistical analysis was performed using SPSS 20.0 software. Orthogonal test data were analyzed by variance analysis. Cell proliferation data were analyzed by one-way ANOVA for inter-group comparisons. If statistically significant differences were found, LSD-t or SNK-q test was used for pairwise comparisons. $P<0.05$ was considered statistically significant.

Results

2.1 Orthogonal Test Results for Preparation Parameters

Following orthogonal experimental design, an L9(3^4) orthogonal array was used to investigate the effects of PVA concentration, PLGA dosage, and ultrasonic power on average particle size, encapsulation efficiency, drug loading, and recovery rate. Nine experimental runs were conducted, and the results are shown in Table 2 .

2.2 Variance Analysis of Orthogonal Test Results

Variance analysis of the orthogonal test results is presented in Table 3 . PVA concentration showed statistically significant effects on the average particle size of BSA nanoparticles ($P<0.05$), while PLGA dosage ($P=0.061$) and ultrasonic power ($P=0.251$) did not, indicating that PVA concentration is the key factor affecting particle size. As shown in Tables 4 and 5 , PLGA dosage had statistically significant effects on both encapsulation efficiency and drug loading ($P<0.05$), suggesting that PLGA dosage is the key factor influencing these parameters. Table 6 demonstrates that PLGA dosage, PVA concentration, and ultrasonic power all had statistically significant effects on recovery rate ($P<0.05$), indicating that all three are key factors affecting nanoparticle recovery.

2.3 Range Analysis and Determination of Optimal Preparation Conditions

According to orthogonal experimental principles, a larger range indicates greater influence of that factor' s level changes on the experimental index. Using smaller average particle size and higher encapsulation efficiency, drug loading, and recovery rate as evaluation criteria, the optimal preparation conditions were determined from Table 7 as: PVA concentration 1%, PLGA dosage 25 mg, and ultrasonic power 70 W. Using these optimal conditions, three batches of BSA nanoparticles were prepared, yielding an encapsulation efficiency of $(73.12\pm6.38)\%$, drug loading of $(4.45\pm0.63)\%$, and recovery rate of $(46.92\pm4.0)\%$.

2.4 Characterization of HGF-Loaded Nanoparticles

Using the optimal conditions, three batches of HGF-loaded nanoparticles were prepared. Transmission electron microscopy revealed regularly spherical nanoparticles with smooth surfaces, exhibiting a membrane-shell reservoir structure encapsulating the drug (Figure 1 [Figure 1: see original paper]). Nanosizer analysis showed HGF nanoparticles had a mean diameter of 234.4 ± 4.8 nm, Zeta potential of -46.72 ± 1.7 mV, and polydispersity index of 0.236 ± 0.012. The size distribution and Zeta potential are shown in Figure 2 [Figure 2: see original paper]. The HGF nanoparticles exhibited an encapsulation efficiency of $(77.75\pm3.04)\%$, drug loading of $(0.002293\pm0.000427)\%$, and recovery rate of $(49.33\pm9.34)\%$.

2.5 In Vitro Release Profiles of Drug-Loaded Nanoparticles

In vitro release studies were performed on both BSA and HGF nanoparticles. As shown in Figure 3 [Figure 3: see original paper], both formulations exhibited three release phases: initial burst release, followed by sustained release, and finally a plateau phase. At 6 h, the cumulative release percentages were $(14.22\pm7.3)\%$ for BSA nanoparticles and $(18.56\pm3.02)\%$ for HGF nanoparticles,

representing the initial burst release. The sustained release phase occurred between 12-120 h. At 24 h, cumulative release percentages were (58.53±4.08)% and (34.65±1.29)% for BSA and HGF nanoparticles, respectively. By 168 h, the values reached (81.72±5.99)% and (79.12±5.36)%.

2.6 Cell Proliferation Assay

The biocompatibility and biological activity of HGF nanoparticles were evaluated using CCK-8 assay. As shown in Figure 4 [Figure 4: see original paper], at 12 h, both HGF nanoparticle and HGF solution groups promoted cell proliferation faster than the control group ($P<0.01$), with no significant difference between them ($P>0.05$). However, at 24 h and 48 h, the HGF nanoparticle group showed significantly stronger proliferative effects compared to the other three groups ($P<0.01$).

Discussion

HGF promotes wound re-epithelialization, induces angiogenesis, and reduces scar formation. However, its short half-life and poor stability limit its efficient utilization. In this study, we used PLGA as a carrier for HGF. The advantages of PLGA include commercial availability of GMP-grade material, stable physicochemical properties, high purity, controlled drug release through stable degradation in vivo, and high biosafety as PLGA hydrolyzes into lactic acid and glycolic acid monomers that can be metabolized through the tricarboxylic acid cycle.

Current methods for preparing PLGA nanoparticles include emulsion-diffusion, salting-out, nanoprecipitation, single emulsion-solvent evaporation (O/W), and double emulsion-solvent evaporation (W/O/W). This study employed the double emulsion-solvent evaporation method, which offers a simpler preparation process, uses less toxic organic solvents, and is suitable for encapsulating water-soluble protein drugs.

To optimize PLGA nanoparticle preparation, we used orthogonal experimental design to investigate the effects of surfactant PVA concentration, PLGA dosage, and ultrasonic power on particle size distribution, encapsulation efficiency, drug loading, and recovery rate. Variance analysis revealed: (1) PLGA dosage is the key factor affecting encapsulation efficiency, drug loading, and recovery rate. This may be because higher PLGA dosage increases oil phase concentration and viscosity, reducing drug diffusion from droplets and thus increasing drug encapsulation. (2) PVA concentration is the key factor affecting average particle size. PVA maintains emulsion stability during preparation, thereby stabilizing nanoparticle morphology and size. Generally, lower PVA concentration results in lower total interfacial area, larger nanoparticles, and vice versa. (3) Both PVA concentration and ultrasonic power are important factors affecting recovery rate. In nanoparticle preparation, surfactants and mechanical forces like ultrasonication are essential for nanoemulsion formation and stability. Insuffi-

cient PVA concentration may cause nanoemulsion droplets to coalesce or break, leading to material precipitation and reduced recovery. Similarly, inadequate ultrasonic power may fail to fully disperse the nanoemulsion, causing droplet coalescence and precipitation, thus reducing recovery. Range analysis identified the optimal conditions as: PVA concentration 1%, PLGA dosage 25 mg, and ultrasonic power 70 W.

Using these optimal conditions, we prepared HGF nanoparticles and evaluated their sustained release capability. The in vitro release profile showed three phases: burst release, sustained release, and plateau. At 6 h, cumulative release percentages were (14.22±7.3)% and (18.56±3.02)% for BSA and HGF nanoparticles, respectively, meeting the Chinese Pharmacopoeia 2010 requirement of less than 40% cumulative release at 0.5 h. The burst release likely resulted from protein drugs adsorbed on the nanoparticle surface immediately diffusing into the release medium. The sustained release phase (12-120 h) occurred because PLGA formed a hydrated membrane after swelling in the release medium, delaying drug release, and as PLGA degraded, drugs gradually diffused into the medium. By 120 h, drug release was nearly complete, with HGF nanoparticles reaching (83.38±3.91)% cumulative release, satisfying the need for rapid exogenous growth factor supplementation during early wound healing.

Finally, cell proliferation assays demonstrated that HGF nanoparticles maintained normal HaCaT cell growth and significantly promoted keratinocyte proliferation, indicating good biosafety and biological activity. At 12 h, HGF nanoparticles showed weaker proliferative effects due to limited HGF release. At 24 h and 48 h, HGF nanoparticles exhibited stronger proliferative effects than other groups, likely because encapsulated HGF was gradually released, increasing local concentration, while HGF in solution was consumed within the first 12 h, reducing its proliferative effect. At 72 h, minimal differences were observed among groups as cells reached confluence.

In summary, this study successfully encapsulated HGF in PLGA nanoparticles with favorable physicochemical properties, achieving sustained HGF release with good biocompatibility and biological activity. However, due to time constraints, biological activity validation was only performed at the cellular level. Future studies will further evaluate biosafety and wound healing effects in mouse skin wound models.

References

[1] Eming SA, Martin P, Tomic-Canic M. Wound repair and regeneration: mechanisms, signaling, and translation[J]. Sci Transl Med, 2014, 6(265): 265sr6.

[2] Maddaluno L, Urwyler C, Werner S. Fibroblast growth factors: key players in regeneration and tissue repair[J]. Development, 2017, 144(22): 4047-60.

[3] Zhang L, Xu P, Wang X, et al. Activin B regulates adipose-derived mesenchymal stem cells to promote skin wound healing via activation of the MAPK

signaling pathway[J]. Int J Biochem Cell Biol, 2017, 87: 69-76.

[4] Werner S, Grose R. Regulation of wound healing by growth factors and cytokines[J]. Physiol Rev, 2003, 83(3): 835-70.

[5] Miyagi H, Thomasy SM, Russell P, et al. The role of hepatocyte growth factor in corneal wound healing[J]. Exp Eye Res, 2018, 166: 49-55.

[6] Bevan D, Gherardi E, Fan TP, et al. Diverse and potent activities of HGF/SF in skin wound repair[J]. J Pathol, 2004, 203(3): 831-8.

[7] Miura Y, Van Ngo Thai Bich, Furuya M, et al. The small G protein Arf6 expressed in keratinocytes by HGF stimulation is a regulator for skin wound healing[J]. Sci Rep, 2017, 7: 46649.

[8] Laiva AL, O' Brien FJ, Keogh MB. Innovations in Gene and Growth Factor delivery systems for Diabetic Wound Healing[J]. J Tissue Eng Regen Med, 2017.

[9] Tayalia P, Mooney DJ. Controlled growth factor delivery for tissue engineering[J]. Advanced Materials, 2009, 21(32/33, SI): 3269-85.

[10] Park JW, Hwang SR, Yoon IS. Advanced growth factor delivery systems in wound management and skin regeneration[J]. Molecules, 2017, 22(8): 1259-79.

[11] Tabata II. The importance of drug delivery systems in tissue engineering[J]. Pharm Sci Technolo Today, 2000, 3(3): 80-9.

[12] 唐宇星, 赵庆, 杨中萌, 等. 聚乳酸共聚物复合脂肪干细胞对骨质疏松性骨折愈后生物力学的影响 [J]. 中国组织工程研究, 2017, 21(10): 1521-6.

[13] 孙雅楠, 张秀梅, 马欣, 等. 红细胞膜包裹 PLGA 纳米粒的制备和体外毒性及药物释放初探 [J]. 中国新药杂志, 2017, 26(19): 2352-7.

[14] 李素铠, 欧阳斌, 徐晓秋, 等. PLGA 纳米药物递送系统的建立及其对 bFGF 生物学活性的维持 [J]. 第三军医大学学报, 2016, 28(11): 1319-23.

[15] Porporato PE, Payen VL, De Saedeleer CJ, et al. Lactate stimulates angiogenesis and accelerates the healing of superficial and ischemic wounds in mice[J]. Angiogenesis, 2012, 15(4): 581-92.

[16] Donaghue IE, Shoichet MS. Controlled release of bioactive PDGF-AA from a hydrogel/nanoparticle composite[J]. Acta Biomater, 2015, 25: 35-42.

[17] Toyoda M, Takayama H, Horiguchi N, et al. Overexpression of hepatocyte growth factor/scatter factor promotes vascularization and granulation tissue formation in vivo[J]. FEBS Lett, 2001, 509(1): 95-100.

[18] Li JF, Duan HF, Wu CT, et al. HGF accelerates wound healing by promoting the dedifferentiation of epidermal cells through beta(1)-Integrin/ILK pathway[J]. Biomed Res Int, 2013: 470418.

[19] Ha XQ, Li YM, Lao MF, et al. Effect of human hepatocyte growth factor on promoting wound healing and preventing scar formation by adenovirus-mediated gene transfer[J]. Chin Med J (Engl), 2003, 116(7): 1029-33.

[20] Chereddy KK, Vandermeulen G, Preat V. PLGA based drug delivery systems: Promising carriers for wound healing activity[J]. Wound Repair Regen, 2016, 24(2): 223-36.

[21] Astete CE, Sabliov CM. Synthesis and characterization of PLGA nanoparticles[J]. J Biomater Sci Polym Ed, 2006, 17(3): 247-89.

[22] Jain RA. The manufacturing techniques of various drug loaded biodegradable poly(lactide-co-glycolide) (PLGA) devices[J]. Biomaterials, 2000, 21(23): 2475-90.

[23] 安森博, 汪龙, 胡懿郃. 聚乳酸-羟基乙酸共聚物载药微球性质及缓释行为的影响因素 [J]. 中国医院药学杂志, 2016, 36(13): 1140-4.

[24] 张晔, 包洪政, 曹洪玉. 表面活性剂聚乙烯醇 (PVA) 在纳米粒子制备中的作用 [J]. 赤峰学院学报: 自然科学版, 2009, 25(1): 15-6.

[25] Landfester K, Eisenblatter J, Rothe R. Preparation of polymerizable miniemulsions by ultrasonication[J]. Jct Res, 2004, 1(1): 65-8.

[26] 王颐婷, 李伟炜, 沈梅, 等. 聚乙二醇/聚己内酯/聚乙烯亚胺载胰岛素纳米粒的体外释药性能 [J]. 南方医科大学学报, 2016, 36(1): 109-15.

Note: Figure translations are in progress. See original paper for figures.

Source: ChinaXiv —Machine translation. Verify with original.