VEGF-conjugated alginate hydrogel prompt angiogenesis and improve pancreatic islet engraftment and function in type 1 diabetes

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ABSTRACT

Type 1 diabetes was a life-long disease that affected numerous people around the world. Insulin therapy has its limitations that may involve hyperglycemia and heavy burden of patient by repeated dose. Islet transplantation emerged as a promising approach to reach periodical reverse of diabetes, however, transplanted islets suffer from foreign body reaction and lack of nutrition and oxygen supply, especially in the blood-vessel-shortage subcutaneous site which was preferred by patient and surgeon. In this study, we designed and synthesized a vascular endothelial growth factor (VEGF) conjugated alginate material to encapsulate the transplanted islets via 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxysuccinimide (EDC/NHS) reaction, and successful conjugation was confirmed by Nuclear Magnetic Resonance H1 spectrum. The best VEGF concentration (100 ng/ml) was determined by the combined studies of the mechanical property and endothelial cell growth assay. In vivo study, conjugated VEGF on alginate exhibited sustained promoting angiogenesis property after subcutaneous transplantation by histology study and islets encapsulated in this material achieved long term therapeutic effect (up to 50 days) in the diabetic mice model. In conclusion, this study establishes a simple biomaterial strategy for islet transplantation to enhance islet survival and function, which could be a feasible therapeutic alternative for type 1 diabetes.

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1. Introduction

Diabetes mellitus (DM) type 1 affects around 285 million people in the world with increasing rate of 3% per year, representing one of the greatest challenges in the medical field [1]. As a conventional treatment strategy, insulin therapy requires careful management of blood glucose to control serious complication and potential hypoglycemia, which brings patient with heavy daily burden [2]. Islet transplantation has been considered as a potentially alternative treatment for type 1 diabetes in spite of major problems related to the acute rejection and short term therapeutic function [3]. Usually patience need administration of immunosuppressive to sustain transplanted islet function but that may cause even more serious problem like allergy, infection and cancer [4].

Islet encapsulation utilized polymer hydrogel as an immuno-protection and islets are enclosed in a matrix surrounded by semipermeable membrane, which allows for the passage of small molecules like insulin and glucose, but keeps away those much larger immune cells and antibodies [5]. Additionally, such a physical barrier can thus prevent allograft rejection and antibody-mediated cytotoxicity, which plays roles in the destruction of beta cells [6]. The most commonly used biomaterial in islet encapsulation is composed of alginate-poly-L-lysine-alginate since its rapid crosslinking property was proved to be plant, when there’s already ischemic damage to transplanted islets [12]. Lots of researchers had been done in order to overcome the delay on revascularization for biomaterials, and among them, incorporation of growth factors was proved to be a promising strategy to strengthen the revascularization and extend the survival for transplanted islets.
testing sample was formed by dropping the pre-gel solution with instron 4502 mechanical tester. VEGF-conjugated alginate hydrogel's compressive strength and modulus on an by calculating the area under curve of its corresponding peak.

In this study, the VEGF-conjugated alginate hydrogel was successfully synthesized and in vivo study indicated that conjugated VEGF was able to provide continuously local stimulation for blood vessels formation after subcutaneous transplantation, which prompted islet function and achieved long term treatment of type 1 diabetes. This study establishes a simple biomaterial strategy for islet transplantation to promote enhanced islet engraftment and function, which was expected to have wildly application on cell therapy.

2. Materials and methods

2.1. Synthesis of VEGF-conjugated alginate hydrogel

Mouse VEGF was conjugated to alginate via 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide/N-hydroxsuccinimide (EDC/NHS) reaction. Briefly, 10 mg alginate powder (Mw = 250 k), 10 mg EDC and 4 mg NHS was added to 1 ml deionization water (DI water) and reacted for 2 h on the ice bath with pH = 5–6. After EDC activation of alginate, VEGF was added (concentration = 1, 10, 50, 100, 500, 1000 ng/ml) to the solution and reacted for 12 h in room temperature with pH = 8.5. Unreacted VEGF and other small molecule were removed by dialysis of the product against DI water in the dialysis tube with cut-off molecular weight = 100 kDa for 3 days. The VEGF-conjugated alginate was concentrated to 1.4% (weight ratio) using ultrafiltration tube with 10 kDa cut-off molecular weight to prepare the pre-gel solution.

2.2. Nuclear magnetic resonance (NMR) H1 spectrum

H1 NMR spectrum was conducted on a Varian Mercury-600 MHz NMR. Alginate and lyophilized VEGF powder were dissolved in Deuterium Oxide (D2O) and H atom of –CONH-bond was quantified by calculating the area under curve of its corresponding peak.

2.3. Mechanical property test

The uniaxial compression was used to measure the VEGF-conjugated alginate gel’s compressive strength and modulus on an instron 4502 mechanical tester. VEGF-conjugated alginate hydrogel testing sample was formed by dropping the pre-gel solution with different VEGF concentration in 20 mM CaCl2, crosslinking for 1 min and equilibrium in DI water. It was then punched and cut into disc form with 2 mm thickness and 8 mm diameter. The cross-head speed was 1 mm/min and six samples were repeated for each test.

2.4. Western blot

VEGF-conjugated alginate hydrogel (100 ng/ml) and free VEGF were run for Western blotting assay, which were loaded in a 12% non-reducing SDS-PAGE and transferred onto a PVDF membrane using an electrophoretic transfer apparatus. Membranes were saturated with 5% nonfat dry milk in TBS and detected with an anti-mouse VEGF mAb. Immunoreactive bands were visualized by enhanced chemiluminescence. Bands were scanned using a scanner (HP Scanjet 7400C, Hewlett-Packard) and their intensities were quantified with the Image J program (NIH).

2.5. Endothelial cell growth assay

To assess the mitogenic activity of VEGF-conjugated alginate, the alginate pre-gel solution with different VEGF concentration were dropped in 20 mmol CaCl2 solution to form gel and equilibrium in PBS to remove small molecule. All samples including pure alginate control were cut into circular discs and put onto the bottom of a 96-well plate. Mice umbilical vein endothelial cells (HUVEC) were seeded onto the gel with 1000/well density in growth media for 24 h. Then the culture media was replaced by M 199 with 10% heat denatured fetal bovine serum (FBS) with another 72 h at 37 °C in a humidified atmosphere with 5% CO2. The cells were then fixed with 10% formalin in neutral buffered solution, followed by May–Gruenwald staining (Sigma). In each experiment, phase pictures of the centerfields of the well were taken using the 4× objective and a Zeiss Axiovert 135 microscope equipped with a digital camera. Cells were counted from printed micrographs. The cell proliferation ratio was calculated by dividing the cell number on the samples by cell number of pure alginate control (presented as 100%).

2.6. Animals

C57BL/6 mice were purchased from the Laboratory Animal Services Center of Hubei University of Chinese Medicine (Wuhan, China). The animals were fed in the new environment (room temperature, 20 ± 1 °C; relative humidity, 55 ± 15%; 12 h light/12 h dark illumination cycle), in which food and water were provided ad libitum throughout the experiments. The mice were treated in accordance with Hubei University of Chinese Medicine (China) animal care guidelines and animal experiments are approved by the Ethical Committee of Hubei University of Chinese Medicine.

2.7. VEGF release assay in vitro

To verify the conjugated VEGF would locally and continuously promote vascularization. Alginate with 100 ng/ml VEGF conjugation was selected for study of VEGF release in vitro and was formed hydrogel as described above. Pure alginate hydrogel incubated with the same concentration (100 ng/ml) of free VEGF was served as control. Both samples and control were transferred to a 24-well plate with 2 ml PBS buffer and incubated in 37 °C. In different time point, the PBS buffer was collected and frozen in −80 °C. VEGF concentration in PBS buffer was measured using mouse VEGF ELISA kit.

2.8. Blood vessel detection in vivo

Algin hydrogel with VEGF conjugation (100 ng/ml) was prepared by dropping the pre-gel solution into CaCl2 solution and equilibrium in sterile PBS to remove small molecule. To verify the benefit of conjugated VEGF, we put a control group by equilibrium the pure alginate gel in PBS containing 100 ng/ml free VEGF. The gel samples were cut into discs with 1 mm thickness and 5 mm in diameter and then subcutaneously implanted in C57BL/6 mice. 1 month after implantation, the gel with its surrounding tissue was collected for tissue sectioning. Blood vessel on the tissue slides was assayed using mouse pan–endothelial cell antigen-32 (MECA-32) antibody staining (BD PharMingen) and the blood vessel density around the implants was calculated.

2.9. Islet encapsulation and transplantation

Islets were isolated from healthy 8–10 week old male C57BL/6J mice (Jackson Laboratories) following standard islet isolation procedures [18]. To establish insulin–dependent diabetic mice model, normal C57BL/6 mice were treated with Streptozocin (STZ) through tail vein. The blood glucose of diabetic mice was retested prior to transplantation. Only mice with non-fasted blood glucose levels were above 300 mg/dl.
for three consecutive days were considered diabetic and underwent transplantation. The mice were anesthetized using 3% isoflurane in oxygen and maintained at the same rate throughout the procedure. After the mice back was shaved, 5 mm incision was cut and gel was subcutaneously transplanted. Wound clips were applied to close the incision and day care monitoring was continued until the clips were removed. The blood glucose of treated mice was monitored 1 time per 3 days. A small drop of blood was collected from the tail vein using a lancet and tested using a commercial glucometer (Clarity One, Clarity Diagnostic Test Group, Boca Raton, FL). Mice with blood glucose levels below 200 mg/dl were considered normoglycemic. Monitoring continued until all mice had returned to a hyperglycemic state.

2.10. Assessment of encapsulated islet function in vivo

The transplanted islet function in vivo was assessed by standard i.v. glucose tolerance test at 15, 30 and 45 days after transplantation. For the stimulation in vivo, 15 mmol glucose solution was injected through the mice tail vein for both experimental and control group. 30, 60, 120, 180 and 240 min after inject, 100 μl blood was collected and centrifuged to obtain the serum. The serum sample was frozen in −80 °C for further analysis. The serum rat C-peptide level was measured by mercodia ultra-sensitive rat C-peptide ELISA kit.

2.11. Statistical analysis

All results are expressed as the means (±S.E.M.) of data obtained from at least three separate experiments. Data in two groups were analyzed by Student’s t-test. All the analyses were performed using the SPSS v 17.0 (SPSS, Inc., Chicago, IL, USA). Differences at P < 0.05 were considered statistically significant.

3. Results

3.1. VEGF-conjugated alginate hydrogel synthesis

The VEGF-conjugated alginate was synthesized by sample EDC/NHS reaction as shown in Fig. 1A, and successful conjugation of VEGF to the alginate polymer backbone was confirmed by HMR (H1) (Fig. 1B). Newly emerged peak in Fig. 1B (b) and (c) with chemical shift equals 7.2 was for the H atom on the –CO–NH–group, which was not presented in the pure alginate polymer in Fig. 1B (a). The area under the curve for the new peak, which indicated the relative VEGF conjugation density, was compared and for reaction concentration of 1, 10, 50, 100, 250, 500 and 1000 ng/ml, and the calculated area was 1.0, 7.8, 58.8, 78.6, 150.9, 314.2 and 688.5, respectively.

3.2. Mechanical property of synthesized VEGF-conjugated alginate hydrogel

The compressive strength reaches the maximum 460.4 KPa when the VEGF concentration was 10 ng/ml. At 50 and 100 ng/ml concentration, the strength was still comparable to the pure alginate hydrogel (Fig. 2A). However, when VEGF concentration was over 250 ng/ml, the strength drastically went down and the alginate was unable to form hydrogel with well-defined shape (Fig. 2A). The VEGF-conjugated alginate hydrogel was also measured for compressive modulus and the modulus has almost the same trend with compressive strength. When concentration equaled 50 and 100 ng/ml, the modulus was the most matched with pure alginate hydrogel and with VEGF concentration increased, the modulus went down drastically (Fig. 2A). However, when the conjugation density increased above 200 ng/ml, both properties drastically decreased and alginate with 1000 ng/ml VEGF could not form gel in good shape and could not be applied for transplantation (Fig. 2B).

3.3. Successful conjugation of VEGF in alginate hydrogel

Western blot was used to assess VEGF protein expression in free VEGF and VEGF-conjugated alginate sample and the results indicated the successful conjugation of VEGF in alginate hydrogel. As Fig. 3A showed, we observed a clear VEGF expression band in the conjugated alginate. Interestingly, little free VEGF could be released from the VEGF-conjugated alginate hydrogel compared with free VEGF according to the molecular weight change (Fig. 3A, lane 3), suggesting the VEGF-conjugated alginate hydrogel provide the biologically active via continuous local stimulation.

The mice umbilical vein endothelial cells growth promotion was the key function of mice VEGF to locally promote new blood vessel formation. The promotion ratio over pure alginate hydrogel was shown in Fig. 3B and as the VEGF conjugation density increased, the promotion ratio exhibited an unexpectedly curved trend. The maximum promotion ratio was reached 146.2% when VEGF concentration equaled 100 ng/ml, and the ratio was going down when VEGF concentration further increased (Fig. 3B).

3.4. Bioactive VEGF-releasing of VEGF-conjugated alginate hydrogel

Chemical conjugation could prevent the quick release of VEGF and enable VEGF to locally promote vascularization. Fig. 4 exhibited the long term release kinetics of VEGF conjugated alginate (100 ng/ml) in PBS buffer under 37 °C. While the control group with free VEGF faded away mostly (90%) within 24 h, the conjugated VEGF could not be
detected to release after 1 day and would release just 30% after one month.

3.5. VEGF-conjugated alginate hydrogel prompt angiogenesis

VEGF conjugated alginate hydrogel (100 ng/ml) was selected for in vivo trail. The hydrogel and surrounding tissue were collected and stained with an antibody binding to MECA-32 in endothelial cells, which was stained brown. Staining images were shown in Fig. 5A and it was quite clear that the surrounding tissue of VEGF conjugated alginate hydrogel was filled with blood vessels in different sizes and the calculated density was 13.87 vessels per 0.1 mm². Normal tissue was also collected for staining (images not shown) and results exhibited that the VEGF conjugated alginate got more blood vessels on the hydrogel/tissue interface than normal tissue. On the contrary, the two control groups of alginate hydrogel with free VEGF and alginate hydrogel showed almost on blood vessels on the hydrogel/tissue interface with calculated density to be 1.66 and 0.47 vessels per 0.1 mm² (Fig. 5B).

3.6. VEGF-conjugated alginate hydrogel enhance islet engraftment and function

Fig. 6A showed the therapeutic curve of encapsulated islet after subcutaneous transplantation. Subcutaneous transplanted islets encapsulated in VEGF conjugated alginate hydrogel (100 mg/ml) could reverse diabetes and control blood glucose below 200 mg/dl for 48 days (n = 8), while islet in the alginate hydrogel with free VEGF could only sustain 12 days (n = 7) and bare alginate hydrogel control just 8 days (n = 6). Moreover, instant C-peptide level after glucose injection revealed the transplanted islet function response to the glucose change and results was exhibited in Fig. 6B. For both alginate hydrogel with free VEGF and bare alginate hydrogel control, the encapsulated islets almost lost their function response to blood glucose change after 15 days of transplantation and therefore lost ability to reverse diabetes. For the alginate with conjugated VEGF, the C-peptide level was obviously...
much higher than the control groups, reaching 0.8 nmol/L at day 30 after transplantation. At day 45 after transplantation, the C-peptide level was lower compared to day 30, corresponding to the therapeutic results that experimental group would about to fail at day 45.

4. Discussion

Alginate hydrogel crosslinked with Ca\(^{2+}\) ion had been widely used for islets encapsulation and transplantation for its quick crosslinking procedure, which was proved to be hospitable to islet survival\[19\]. However, few studies could achieve long term therapeutic effect when islets were transplanted subcutaneously. One reason is that compared with other transplanted site like abdomen and portal vein, subcutaneous site was short of available blood accessibility and oxygen supply, leading to the poor engraftment of transplanted islets\[20,21\]. Therefore VEGF was incorporated into the alginate hydrogel platform to improve the vascularization for the transplants. Instead of encapsulating the VEGF and letting it release, like many other researchers did, we conjugated the VEGF to the alginate backbone and expected the VEGF would locally promote vascularization for a long term. The conjugation was achieved by EDC/NHS reaction and the newly formed \(-\text{CO} \equiv \text{NH}-\) group, which was not presented in alginate polymer backbone, could be utilized to confirm successful conjugation. We previously planned to determine the precise conjugation density with different VEGF react concentration through comparing the NMR area under the curve. However, the peak for H atom on the alginate polymer was irregular and the area was hard to precisely determine. Relative conjugation density was calculated for different react concentration and at low concentration, the relative conjugation density was almost the same with react concentration. However, with the react concentration increased, the conjugation density was obviously lower compared to the react concentration, which was caused by the steric effect of large VEGF molecule.

Alginate hydrogel was known for its robust mechanical property and modulus comparable to tissue, which was crucial for successful tissue integration\[22\]. With the help of VEGF to locally promote blood vessel to supply nutrition and oxygen to encapsulated cell, the alginate hydrogel could be a superior platform for cell therapy, therefore we expected to incorporate as much as VEGF to the alginate backbone without hindering the mechanical property and modulus of the hydrogel. When VEGF conjugation density was below 100 ng/ml, both compressive

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**Fig. 5.** VEGF-conjugated alginate hydrogel prompt angiogenesis. (A) Blood vessel formation in tissues near subcutaneously implanted with VEGF-conjugated alginate hydrogel (100 ng/ml), alginate hydrogel with free VEGF and alginate hydrogel following MECA-32 staining. A representative experimental result was shown (\(n = 6\)). (Black asterisk indicated the hydrogel site). (B) Quantification and comparison of blood density among VEGF-conjugated alginate hydrogel (100 ng/ml), alginate hydrogel with free VEGF and alginate hydrogel (***\(P < 0.001\)) (\(\pm\)S.E.M. \(n = 6\)).

**Fig. 6.** VEGF-conjugated alginate hydrogel enhance islet engraftment and function. (A) Therapeutic results of islet encapsulated with VEGF-conjugated alginate hydrogel (100 ng/ml) and control group in diabetic mice (\(\pm\)S.E.M. \(n = 6\)). (B) Rat C-peptide level among VEGF-conjugated alginate hydrogel group in different day and control group for 15 days (\(\pm\)S.E.M. \(n = 6\)).
strength and modulus of VEGF alginate hydrogel were on the same level of pure alginate hydrogel, which is suitable for transplantation. However, when the conjugation density increased above 200 ng/ml, both properties drastically decreased and alginate with 1000 ng/ml VEGF could not form gel in good shape and could not be applied for transplantation. Considering the mechanism of alginate crosslinking, when the VEGF conjugation density was very high, the steric effect of large VEGF molecule decreases the crosslinking point between alginate polymer chains, as shown in Fig. 7. At the same time, the COOH group, which was crucial for Ca\(^{2+}\) crosslinking, was occupied and therefore prevents the hydrogel formation.

VEGF was known for promoting the umbilical vein endothelial cell growth and therefore enhances the local vascularization [23–25]. We previously assumed that the higher the VEGF conjugation density on alginate, the more promotions of cell growth would occur on alginate hydrogel. However, the promotion ratio to the bare alginate hydrogel also showed curve-like unexpectedly and the same experiment was done several times to confirm the result. We assume the decrease in proliferation ratio was also due to the hydrogel crosslinking property (discussed above). When the conjugation density was low, the alginate polymers were well crosslinked and no free polymer would hinder the growth of the cell but when conjugation density was high, the alginate polymers were poorly crosslinked and there might be free alginate polymer in locally high concentration, which was toxic to cell and hinder the cell growth. Both the mechanical property and cell promotion results indicated 50 and 100 ng/ml conjugation density were superior therefore we selected 100 ng/ml VEGF conjugated alginate for further in vivo application.

Due to vascularization promotion property, VEGF was wildly utilized in engineering cell encapsulation biomaterial for transplantation. However, most of the researches just incorporate free VEGF or encapsulated VEGF to reach controlled release after transplantation [26,27]. Most of biomaterial would induce foreign body reaction when subcutaneously transplanted and would be encapsulated by a thick layer of fibrosis [28,29]. It was crucial that blood vessel would penetrate through the fibrosis and sustain encapsulated cell survival [30]; therefore local blood vessels were more important than vessels far away from material [31]. By conjugating the VEGF to the biomaterial matrix, VEGF could locally promote vascularization in the tissue around the transplanted hydrogel. Free VEGF with the same concentration of reaction in the hydrogel would fade away within 24 h and not be able to promote vascularization in a long term while in vitro release showed few of conjugated VEGF would release after one month incubation. Also in the study of Western blot, little free VEGF could be released from the VEGF-conjugated alginate hydrogel. It is possible that non-conjugated VEGF or treatment in Western blotting progress might contribute to the extremely little VEGF. Important, the results confirm on the other side that the VEGF-conjugated alginate hydrogel provide the biologically active via continuous local stimulation.

Base on previous studies, 100 ng/ml conjugation density sample was utilized for in vivo test. VEGF was known for promotion of blood vessel formation and the calculated blood vessel density clearly showed that the VEGF conjugated alginate hydrogel induced more blood vessel formation and the density was even higher than normal tissue. Higher density of blood vessels provides enough oxygen and nutrition to the implant and sustains survival of encapsulated cell for therapy. The alginate hydrogel with free VEGF got almost no blood vessels on the tissue/implants interface and based on the result of in vitro release experiment, quick release of VEGF to the surrounding tissue would not result in blood vessel formation. Instead, released VEGF would be metabolized and there would be no promotion effect anymore. The conjugated VEGF was able to locally promote vascularization for long term and finally result in abundant vessels in hydrogel/tissue interface.

Therapeutic effect was the most effective way to examine the transplanted cell function and the superiority of biomaterial. Islets encapsulated in VEGF conjugated alginate hydrogel were able to reverse diabetes and control blood glucose for about 50 days, which was far longer compared with the two control groups. Rat C-peptide level after glucose injection also revealed that two control groups had almost no functional rat islets 15 days after transplantation, which were killed by shortage of nutrition and oxygen supplied by blood. For VEGF conjugated hydrogel, we found that the encapsulated islets functioned best at day 30 after transplantation when blood vessels were fully grown with instant blood accessibility. When we explanted the hydrogel after every trail ended, there were still lots of blood vessels surrounding the implant. Therefore we suspected that even with sufficient blood supply, xenografted rat islets were finally erased by immune cells coming along with the blood. Nevertheless, the VEGF conjugated alginate was superior in subcutaneous transplantation with few other researches could achieve long term effect.

5. Conclusions

Taken together, we have synthesized VEGF-conjugated alginate for islets encapsulation and transplantation in this study. VEGF-conjugated alginate hydrogel was studied for mechanical and cell proliferation properties. The best VEGF conjugation density was selected through the combined analysis of properties and in vitro release showed long term stability of conjugated VEGF, which would locally promote vascularization in the body. In vivo trial in normal mice proved that abundant blood vessels would form in the tissue around the implanted VEGF-conjugated alginate hydrogel. Therapeutic effect to reverse diabetes in diabetic mice model revealed the potential application of the VEGF-conjugated alginate hydrogel for long-term subcutaneous islet transplantation. We hope that the simple biomaterial strategy could benefit the fight to cure type 1 diabetes.

Disclosure statement

The authors declared no competing interests exist.

Author contributions

N.N.Y., H.Y.M., H.L.X and Z.B.C. designed the research. H.L.X synthesized the materials. N.N.Y. and H.Y.M. finished the experiments and wrote the manuscript. H.L.X., Y.S.G., T.Y. and J.L.Y. performed partial research. L.D. and D.J.C helped to analyze the data. H.L.X. and Z.B.C. revised the manuscript.

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