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Evaluation of Angiogenic Factor Release from Thermosensitive Poly(N-Vinylcaprolactam)-g-Collagen: In Vitro and In Vivo Studies

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ABSTRACT

In this study, a thermosensitive poly(N-vinylcaprolactam)-g-collagen (PNVCL-g-Col) hybrid hydrogel was synthesized by conjugation using the NHS/EDC cross-linking system, and characterized. At first, the efficiency of in vitro sustained delivery of human vascular endothelial growth factor (VEGF) from the thermosensitive PNVCL-g-Col hydrogel modified with heparin, was evaluated for duration of ten days under in vitro physiological conditions (37°C, pH 7.4). The results indicated that PNVCL-g-Col hydrogel preserved its stability and released ~90% of the loaded VEGF within this time period. In vitro study showed that PNVCL-g-Col was basically histocompatible. Then, the in vivo angiogenic activity of the VEGF-releasing PNVCL-g-Col was investigated using a subcutaneous rat model. In vivo study confirmed that angiogenic-factor-loaded PNVCL-g-Col had the capacity to induce neovascularization indicating that the in vivo bioactivity of the VEGF was preserved in the thermosensitive PNVCL-g-Col.

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INTRODUCTION

Intelligent hydrogels which are sensitive to an external stimulus, such as temperature, pH, light, etc. rapidly change their physical structure (size, shape, solubility etc) in response to the stimulus [1-3]. Thermosensitive hydrogels are among the most widely employed intelligent hydrogels. They are characterized by their low critical solution temperature (LCST) [4], and exhibit a distinct and highly reversible temperature-triggered behaviour. Poly (N-vinylcaprolactam) (PNVCL) is a thermosensitive synthetic polymer with a LCST of ~32°C, which is near the range of physiological temperature [5,6]. It is in a swollen state at a temperature below the LCST; however the polymer networks collapse at temperatures above the LCST. Unlike the well-known thermosensitive polymer PNIPAM, hydrolysis of PNVCL does not produce toxic amide compounds making it attractive for biomedical and pharmaceutical applications.

Collagen as a biodegradable and biocompatible natural polymer is the most abundant structural protein in the extracellular matrix (ECM) [7]. Due to its excellent properties, there is great interest in using collagen-based biomaterials for tissue engineering and regenerative applications. However, one of the problems of using collagen hydrogels is their poor mechanical properties. Collagen can be conjugated with other polymers using carbonyl moiety (-C=O), amine groups (-NH_2/-$CONH_2$ amide bonds), and hydroxyl groups (-OH) as side groups.

The combination of a thermosensitive synthetic polymer and a natural polymer (such as, collagen, hyaluronic acid, or fibrin) is defined as a hybrid polymer, and may have the potential to produce biomimetic scaffolds with desirable mechanical, biocompatibility, and other properties. Such hybrids could have potential applications in the broad fields of biomedicine [8], biotechnology, and tissue engineering [9].

Fundamental challenges associated with biomimetic scaffolds are lack of a microvascular system which is essential for certain tissues to maintain their viability and function in terms of the transport of nutrients and the signal molecules. Vascular endothelial growth factor (VEGF) is a major regulator of the neovascularization (angiogenesis). Daily injections of angiogenic factors are not suitable due to their short half-lives; thus it is necessary to incorporate the growth factors into hydrogels for

their use [10]. Promotion of localized angiogenesis via sustained release of growth factors from hydrogel systems is an attractive field [11].

In this study, a novel thermosensitive PNVCL-g-Col copolymer was synthesized, and evaluated for its potential as a carrier for the sustained release of an angiogenic growth factor. The thermosensitive growth factor release was investigated in terms of the change in the hydrophilic-hydrophobic character, gel-forming and swelling properties of the hybrid biopolymer. The thermosensitive PNVCL-g-Col hydrogel underwent reversible structural transitions from a closed state to an open state with the help of external temperature stimuli, giving on-off switches to modulate growth factor delivery.

MATERIALS AND METHODS

Chemicals

N-Vinylcaprolactam (NVCL), EDC, NHS, and 3-mercaptopropionic acid (MPA) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2,2'-Azoisobutyronitrile (AIBN) was supplied from Acros Organics (Thermo Fisher Scientific, Geel, Belgium). All other chemicals and solvents were supplied from either Sigma or Merck (Darmstadt, Germany).

Synthesis of PNVCL with Carboxyl End Group

Thermosensitive PNVCL-COOH was synthesized by radical polymerization in solution [12]. The mixture of monomer NVCL (37.35 mmol), MPA (3.278 mmol) in 50 mL of pure ethanol was added to a sealed flask. The oxygen was removed from the reaction solution through dried nitrogen for 20 min. Then, the initiator AIBN (0.304 mmol) was added to the reaction mixture. Polymerization was performed under nitrogen atmosphere at 75°C for 8 h. Samples were precipitated in diethyl ether. Upon drying in vacuum, the solid was re-suspended in ultrapure water. Then, the solution was dialyzed for 3 days against ultrapure water in cellulose membrane tubing (MW cutoff 1000 Da), followed by lyophilization.

Synthesis of the copolymer

Collagen (isolated from rat-tail tendon; Sigma) solution was prepared in 0.1% acetic acid. Ten milliliter collagen solution (1% w/v) was mixed with aqueous PNVCL-COOH solution. Later, 0.1 mL solution of NHS (0.023 g) and EDC (0.0384 g) in ultrapure water was introduced (PNVCL-COOH/Collagen; 5:1). The reaction was performed under constant stirring for 4 h at room temperature.

Fabrication of PNVCL-g-Col Constructs

In order to obtain PNVCL-g-Col constructs, the PNVCL-g-collagen solution was individually dropped onto the wells of a 24-well cell culture plate (1 mL/well each) and frozen overnight at -80°C. The solidified PNVCL-g-Col was then transferred into a freeze-drying vessel (Alpha1-4 LD Plus, Christ, Osterode am Harz, Germany) under vacuum (<20 Pa) at -60°C temperature and freeze-dried for 24 hours. Finally, PNVCL-g-Col hydrogel constructs were obtained.

ESEM Analysis

Morphology of the lyophilized PNVCL-g-Col construct was analyzed by environmental scanning electron microscopy (ESEM). The samples were coated with a thin layer of gold and analyzed under a (FEI Quanta 200 F FEG) environmental scanning electron microscope.

Angiogenic Factor Loading and in Vitro Release Study

PNVCL-g-Col hydrogel constructs were incorporated with the angiogenic factor, VEGF (from Sigma). Loading of VEGF on PNVCL-g-Col was carried out as follows: 200 µL of heparin (0.5 %) was slowly dropped onto each dry PNVCL-g-Col construct and incubated for 2 hours at 4°C and then 400 ng of VEGF in 200 µL of PBS with 1 mg/mL BSA was added dropwise onto each construct and incubated overnight at 4°C. Heparin was immobilized by the free carboxyl groups of PNVCL-g-Col copolymer, and then VEGF was bound to heparin, since it is a heparin-binding growth factor. All the procedures were conducted under aseptic conditions.

In vitro release of VEGF from thermosensitive PNVCL-g-Col was performed under static conditions. Briefly, the VEGF-loaded PNVCL-g-Col was immersed in 1 mL of 0.1 M PBS (pH 7.2) and incubated at 37°C. The release medium was removed (400 µL) and replaced with fresh buffer at 3, 6, 9, 12 and 24 h, and at days 2, 3, 7, 10, 14 and 21. The amount of released VEGF throughout of the PNVCL-g-Col hydrogel construct (n=3) was quantified by an VEGF enzyme-linked immunosorbent assay (Thermo-Fisher) using a spectrophotometer at 450 nm. The experiments were performed in triplicates.

In Vivo Study

A total of 4 adult male Wistar rats (200-250 grams in weight) were used in this study. The animals were exposed to 12 h of light followed by 12 h of dark, temperature (24°C) and humidity (50%), were supplied with unlimi-

ted drinking water and food on daily basis for 14 days. At implantation, the rats were anaesthetized by Avertin (Sigma), their abdominal regions were shaved, swabbed with 70% isopropanol and then Wescodyn. Using a sterile surgical set-up, the animals were draped and an incision of ca. 3 cm in length was made on the skin and the PNVCL-g-Col constructs (ca. 0.5 g) loaded with 200 µL of heparin (0.5 %) and 200 µL of VEGF (200 ng for each construct) were placed in the right and left groin fascia. The skin was sutured after the addition of 0.1 mL sodium cefazolin antibiotic, and closed using 9 mm wound clips (Becton Dickinson, Sparks, MD). The implants were carefully explanted with some of the surrounding tissue after 14 days, fixed, embedded in paraffin, and analyzed by histochemistry.

Statistical Analysis

All quantitative experiments were performed in triplicates. Results were expressed as mean ± standard deviation, and evaluated with one-way analysis of variance. Statistical significance value was set at $P < 0.05$.

RESULTS AND DISCUSSION

The grafting reaction of the PNVCL-g-Col (Fig. 1(A)) was carried out by amidation of the collagen amine groups with the acid groups of the PNVCL-COOH, using crosslinking agents (EDC/NHS) at room temperature. The PNVCL-g-Collagen hydrogel constructs with approximately 15-mm diameter (h=5 mm) were formed using the PNVCL-g-Collagen solution in 24 well culture plates by the lyophilization method, and stored at room temperature until further use (Fig.s 1(B) and 1(C)).

PNVCL-g-Col exhibited a phase transition at around 38°C. The PNVCL-g-Col was transparent below the LCST, and became white and opaque due to a higher temperature than the LCST (Fig. 2).

The higher thermal transition temperature of PNVCL-g-Col copolymer when compared with PNVCL-COOH was attributed to the increase in hydrophilic groups of the collagen. In connection with the new hydrogen bond formation between water molecules and the polar functional groups in the collagen, it is necessary to reach higher temperatures to remove water from the polymer network. The chemical structure and grafting level of the newly-formed copolymer directly influence the LCST of the thermosensitive hydrogel [13]. Other studies with thermosensitive PNIPAAm have shown that the presence of proteins increase the hydrophilicity of the polymer-protein complex [14].

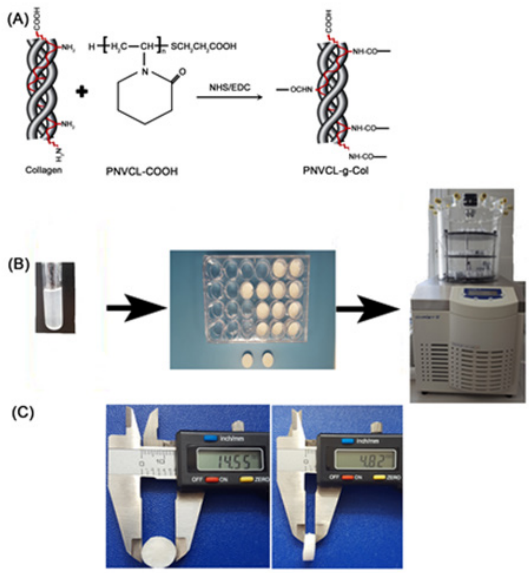

Figure 1. (A) Chemical structure of crosslinked PNVCL-g-Col. (B) Fabrication of PNVCL-g-Col scaffold by lyophilization. (C) The fabricated scaffolds had dimensions of ~15 mm in diameter and 5 mm in thickness.

In vitro VEGF Release

PNVCL-g-Col constructs exhibited repeated and reversible swelling-shrinking behavior in aqueous solution depending on environmental temperature. Findings indicate that below the LCST, PNVCL-g-Col copolymer was in a swollen state, while above the LCST an insoluble white-colored form was generated due to the collapse and shrinkage of the PNVCL-g-Col network. Previous studies based on the controlled release of bovine serum albumin and lidocain from PNVCL-g-Col have shown a release behavior in relation to the change in the LCST [15].

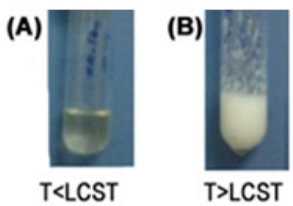

Figure 2. Phase-transition behavior of thermosensitive PNVCL-g-Col: (A) soluble form below LCST. (B) insoluble form above LCST.

The time course of cumulative and daily VEGF release from PNVCL-g-Col hydrogel was invezstigated at 37°C and the results are presented in Fig.s 3(A) and (B), respectively. According to the in vitro cumulative release results, an initial burst VEGF release was observed at the first 24 hours. The release rate slowed down gradually till the end of 72 hours. A daily in vitro release rate of ~1000-200 pg/ml VEGF was obtained; thus this daily dose was later found to be sufficient to support neovascularization in vivo. From

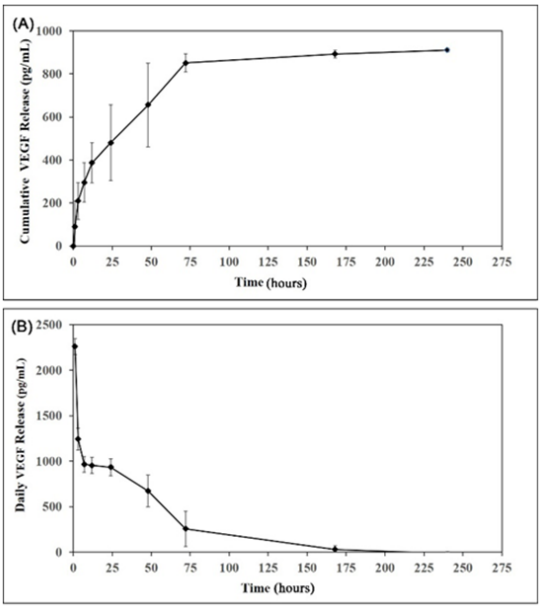

Figure 3. (A) Cumulative in vitro release, and (B) daily in vitro release of VEGF from PNVCL-g-Col.

day 4 to 7, VEGF release had at a slow steady-state phase. After seven days, release of VEGF from PNVCL-g-Col had significantly decreased.

In a study, the VEGF release from a combination of poly(lactide-co-glycolide) (PLGA) microspheres embedded in PLGA matrix was investigated during one month [16]. VEGF release from embedded microspheres was found to be 197 ng/ml which was an initial burs,t followed by release at a significantly lower amount (1.1 ng/ml) [16]. In another study, it was reported that VEGF (2.5 and 5.0 μg) released from activated PLGA sponges displayed a burst like release (~48% first day) profile at the first 3 days (~72%) and observed a relatively slower VEGF release later [17].

We used heparin modification, in order to provide a suitable binding site for the growth factor; as known, VEGF is a heparin-binding growth factor. In another study, hydrogels composed of chemically-modified hyaluronan (HA) and gelatin were used as for the sustained release of human VEGF, in vitro and in vivo [18]. In this particular study, heparin was incorporated similarly with the hydrogel for obtaining a prolonged and greater angiogenic response.

Apparently, the major factor responsible for the decrease in the diffusion of bioactive agents from a hydrogel is its phase transition from the swollen state at low temperatures, to the compact state at high temperatures. The morphology of the collapsed of PNVCL-g-Col shows a reduced pore size, and as a result of this condition free diffusion is prevented leading to the slow release of the growth factors at physiological temperature. VEGF was not completely released from the PNVCL-g-Col hydrogels after 21 days duration. One possible reason for this could be the presence of interactions between the proteins and the PNVCL-g-Col membranes.

ESEM Analysis

The PNVCL-g-Col demonstrated a distinctly different appearance with physical changes evident even at the macroscopic level, including the color and rigidity depending on temperature. The surface and internal morphology of the PNVCL-g-Col had different microstructure. PNVCL-g-Col constructs had a smoother structure with lesser pores. (Fig. 4). ESEM of the lyophilized PNVCL-g-Col construct exhibited a relatively homogeneous and ordered structure, highly porous and pores connected with each other. Pore structure, size and distribution of the hydrogel constructs are known to influence the equilibrium swelling ratio, the release profile, mobility and mass transfer.

In Vivo Study

The in vivo angiogenic effect of the VEGF-loaded PNVCL-g-Col construct was evaluated in the Wistar rat subcutameous model for control and experimental treatment cases, including (i) HA-loaded PNVCL-g-Col, and (ii) HA-VEGF loaded PNVCL-g-Col (Fig. 5). In brief, signs of significant inflammation were not observed, and the polymer conjugates were quite well-tolerated by the subjects. Histology showed that the hydrogel was well tolerated and could be defined as histocompatible, as also

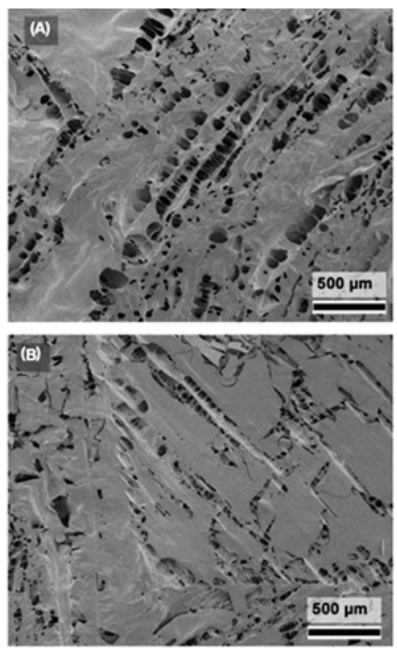

Figure 4. SEM micrographs of freeze-dried PNVCL-g-Col: (A) surface, (B) cross-section (scale bars= 500 μm).

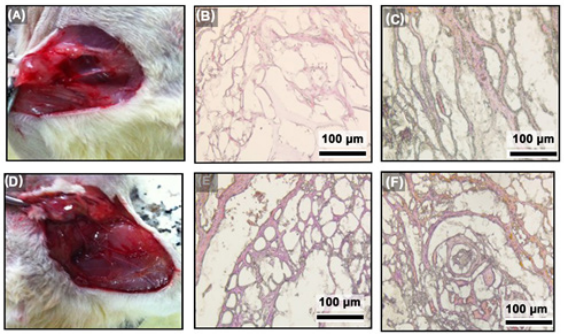

Figure 5. Macroscopic images of VEGF-releasing hydrogel explants 1 weeks (A) and 2 weeks (D) post-implantation. Histochemical findings (H&E-stainings): (B) Col, (C, E) PNVCL-g-Col-VEGF after 7 days, and (F) PNVCL-g-Col-VEGF after 14 days. Scale bars= 100 µm.

shown previously [15].

H&E stainings indicated that HA-VEGF loaded PNVCL-g-Col scaffold induced in vivo neovascularization. The newly-formed vessels could be observed in the H&E histology sections retrieved at 2 weeks. This findings support that the released VEGF from the PNVCL-g-Col had preserved its angiogenic activity, which is mandatory for neovascularization [19].

CONCLUSION

A thermosensitive PNVCL-g-Col hybrid construct was fabricated by chemical copolymerization, and lyophilization. Incorporation of the angiogenic growth factor VEGF was successfully achieved. The hybrid hydrogel supported in vitro sustained release of VEGF. PNVCL-g-Col exhibited a sustained release depending on temperature. The in vivo angiogenic effect was achieved by the VEGF-releasing thermosensitive hydrogel as well. This hybrid thermosensitive hydrogel may have potential for n neovascularization therapies and tissue engineering, in the future.

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