Thermoresponsive and Biodegradable Hydrogels for Sustained Release of Nerve Growth Factor to Stimulate Neurite Outgrowth

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Summary: A series of thermoresponsive and biodegradable hydrogels were developed for sustained release of nerve growth factor (NGF) for ultimate goal of treating neurological disorders. These hydrogels were composed of poly(*N*-isopropylacrylamide) (PNIPAAm) as a thermoresponsive unit, poly(L-lactic acid) (PLLA) as a hydrolytically degradable and hydrophobic unit, and dextran as an enzymatically degradable and hydrophilic unit. NGF was loaded into these hydrogels in aqueous solution with various loading efficiencies depending on the compositions of hydrogels. *In-vitro* release study showed that the release of NGF from these hydrogels could last for at least 15 days. NGF released from the hydrogels was able to stimulate neurite growth from PC12 cells.

Keywords: biodegradable; hydrogel; *N*-isopropylacrylamide; nerve growth factor; neurite outgrowth; PC12 cells; thermoresponsive

Introduction

Neurological damages in the central nervous system caused by traumatic brain injury and brain diseases such as Parkinson disease and Alzheimer's disease can disrupt the receiving and transmission of information through the body, thus affecting patient's motor, sensory and autonomic functions.[1,2] Currently, there is no cure for such patients since the lost functions could not be completely restored using current clinically available treatments.[3] However, with the advances in regeneration medicine and the understanding of the disease processes, it is very promising that the lost or damaged functions might be restored through therapies such as delivery of neural therapeutics via engineered scaffolds or delivery devices.[2–7] Recently, neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3 and neurotrophi-4 have been investigated as therapeutics to treat nerve injuries and brain diseases.[1,2,8–12] However, like other protein/peptide drugs, how to efficiently deliver neurotrophic factors remains a big challenge since they do not readily cross the blood-brain barrier, have a short half-life, and are easily metabolized at other tissue sites.

To address the above issues, polymeric devices like hydrogels and electrical spin fiber mats have been designed for controlled delivery of neurotrophic factors.[1,13,14] Hydrogels are physically or chemically cross-linked polymers, which absorb a large amount of water (at least 20 wt% of the weight of dry polymer) and meanwhile maintain their three-dimensional solid shapes. The network structures of hydrogels can be tailored to meet specific requirements in the mechanical, responsive, diffusive, hydrophilic and hydrophobic, surface tension and wetting properties.[15–17] Hydrogels can also protect therapeutic peptides and proteins from harsh conditions. All these advantages as well as their high water content, rubbery nature, and good biocompatibility[18–20]

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make them good candidates as drug delivery systems and scaffolds for cell transplantation. In particular, poly(NIPAAm) (PNIPAAm)-based polymers and hydrogels have been frequently studied as drug delivery systems. Covalently crosslinked PNIPAAm hydrogels experience a sharp phase transition at the LCST (i.e. around 32 °C) in aqueous environments with the property alternating between highly hydrophilic and hydrophobic at temperatures below and above the LCST, respectively. Therefore, this thermo-responsive property enables efficient drug loading in aqueous solutions at low temperatures, thus avoids organic solvents and helps prevent proteins from denaturation. Through copolymerizing with other hydrophilic or hydrophobic monomers,[21–23] the physical, chemical, and mechanical properties of PNIPAAm-based hydrogels can be improved or adjusted. Therefore, drugs with a wide range of hydrophobicity and hydrophilicity may be loaded into these hydrogels under mild conditions. Previously, our research group has successfully copolymerized NIPAAm with hydrophobic and biodegradable poly(L-lactic acid) (PLLA), and hydrophilic dextran to obtain thermo-responsive and biodegradable hydrogels for controlled drug delivery and cartilage tissue engineering.[24–26] In present study, we further explore these thermo-responsive and biodegradable hydrogels for sustained release of NGF. NGF was loaded into these hydrogels in aqueous solutions and the release of NGF from hydrogels was studied over a period of 35 days. PC12 cells were used as a neuronal cell model to evaluate the cytotoxicity of these hydrogels and the bioactivity of NGF released from these hydrogels.

Experimental Part

Materials

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO), including dichloromethane (MC), dimethylformamide (DMF), bovine serum albumin (BSA), nerve growth factor (NGF), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2-propanal, hexane, N,N,N′,N′-tetramethylethylenediamine (TEMED), 1-Hydroxybenzotrazole (HOBt), 1,3-dicyclohexylcarbodiimide (DCC), 2-aminoethanol, acryloyl chloride, allyl isocyanate, triethylamine, 2,2-azobis(2-methylpropionitrile) (AIBN), sodium dodecyl sulfate (SDS), L-glutamine, penicillin/streptomycin, trypsin, phosphate buffered saline (PBS), N-isopropylacrylamide (NIPAAm) and N-N-methylene-bis-acrylamide (BIA). PLLA (MW 2000 $g \cdot mol^{-1}$) was obtained from Polyscience (Warrington, PA). Dextran (MW 1500 $g \cdot mol^{-1}$) was ordered from Fluka (Switzerland). RPMI 1640 was purchased from American Type Culture Collection (Manassas, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). Heat-inactivated horse serum was ordered from Invitrogen (Grand Island, NY). All these chemicals were used as received unless otherwise indicated. Deionized distilled water was used in all the experiments. NIPAAm was purified by recrystallization from hexane before use.

Synthesis of Macromers

Photopolymerizable macromers, acrylated PLLA and allyl-dextran macromer, were synthesized from PLLA and dextran, respectively. Details about the macromer synthesis and the characterization of these macromers can be found in a previous publication from our group.[25] Briefly, PLLA polymers with hydroxyl groups at one end and carboxylic acid groups at the other end were converted into polymers with hydroxyl groups at both ends by reacting PLLA with 2-aminoethanol using DCC as a coupling agent. After purification, the resulting PLLA diols were reacted with aryloyl chloride to obtain acrylated PLLA with acryloyl groups at both ends. The allyl-dextran macromer was obtained by reacting dextran with allyl isocyanate in DMF with triethylamine as a catalyst. The degree of substitution in the allyl-dextran macromer is 28.5 per 100 dextran glucopyr-

anose residues, which means that each allyl-dextran macromer has about 2.6 C=C double bonds. In this case, both acrylated PLLA and allyl-dextran macromers function as co-monomers as well as crosslinkers for the following syntheses of the thermoresponsive and biodegradable hydrogels.

Synthesis of Hydrogels

Acrylated PLLA macromer, allyl-dextran macromer, and NIPAAm were dissolved in DMF at 1 g/4.5 ml DMF according to Table 1. Then BIA was added at 4 mol%, based on a total amount of C=C double bonds of NIPAAm monomers, acrylated PLLA macromers, and allyl-dextran macromers. Dry nitrogen was used to purge the above solution for about 30 min. AIBN (2 mol% of the total C=C double bonds) in 0.5 mL DMF was added into the above solution, followed by feeding of TEMED (6.4 mol % of the total C=C double bonds). The solution was continuously purged using nitrogen for 5 min and then transferred into a mold consisting of a Teflon® frame (3 mm thick) as the spacer and two glass plates previously treated with Pro-Sil solution to aid mold release. The mold was sealed and kept at 65 °C for 4 h. Disk-shaped samples (8 mm in diameter) were cut from the resulting hydrogel films. The disk-shaped hydrogel samples were then washed using a 50:50 ethanol/water (v/v) cosolvent for several times within 24 h. The washed samples were then dried for several days until no further weight loss. The structure of synthesized hydrogels is shown in Scheme 1. Same procedure was used to synthesize PNIPAAm homopolymer hydrogels. The thermoresponsive, dynamic swelling and degradation properties of hydrogels have been characterized and reported in two publications from our group.[25,26]

Table 1.
Composition for hydrogel synthesis.

Hydrogel	Monomer/ Macromer Feeding Molar Ratio		
	NIPAAm	L-lactic acid unit in acrylatedPLLA	Glucose unit in allyl-dextran
S-80-15-5	80	15	5
S-90-10-0	90	10	0
S-93-5-2	93	5	2

SEM Characterization of Hydrogels

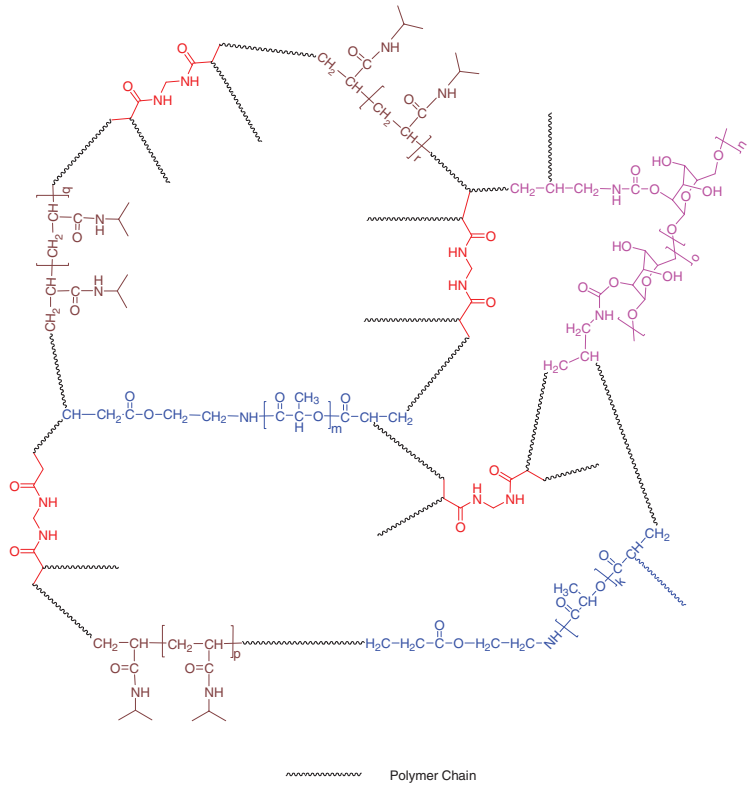
Scanning electronic microscopy (SEM, Hitachi S-3500N) was used to analyze hydrogel surface morphologies. Dry hydrogel samples were incubated in PBS (pH 7.4) solution at 37 °C. At days 4 and 40, the samples were removed from the PBS solutions and immediately dipped into liquid nitrogen to retain the swollen structure followed by lyophilization for overnight. The lyophilized sample was coated with gold for SEM study. Unswollen hydrogel samples dried in the air were used as control for time zero.

Release Kinetics of NGF from Hydrogels

For NGF loading, dry hydrogels S-80-15-5, S-90-10-0, and S-93-5-2 were soaked in 2 ml (8 hydrogels) of 25 $\mu g \cdot ml^{-1}$ NGF PBS solution (pH 7.4, containing 1 w/v% BSA) at 10 °C. One day later, the hydrogels were removed from the aqueous solutions and freeze-dried. NGF left in the aqueous solutions was quantified using NGF ELISA assay.[13,14] To study the NGF release kinetics from the hydrogels, each dry NGF-loaded sample was immersed in 2.5 ml BSA/PBS solution (pH 7.4) at 37 °C. At selected time points, 1 ml release solution was taken out for NGF concentration analysis and replaced with 1 ml of fresh PBS/BSA solution (pH 7.4) to maintain the total volume of release medium (2.5 ml). NGF ELISA assay was used to quantify the amount of NGF released in the media.[13,14]

Cytotoxicity of Hydrogels

PC12 cells were seeded in 24-well plates at 75,000 cells/well in 0.5 ml RPMI 1640 medium containing 5% fetal bovine serum, 10% heat-inactivated horse serum, 3.6 mM L-glutamine, and 1% penicillin/streptomycin (cell density is 150,000 cells per ml), and incubated in a humidified incubator under a 5% CO_2 atmosphere at 37 °C for 24 h.



Scheme 1.
Chemical structure of hydrogels containing NIPAAm, PLLA, and dextran components with BIA as a crosslinker.

Sterilized hydrogels S-90-10-0, S-93-5-2, or S-80-15-5 without NGF loading were then added and incubated with the cells in each well. Cell viabilities were determined using MTT assay[27] after the hydrogels were incubated with PC12 cells for 1 and 6 days. For 6-day incubation, each culture medium was changed by half with fresh medium on the 4th day.

Biological Activity of NGF Released from the Hydrogels

PC12 cells were plated onto collagen-coated 48-well at a density of 20,000 cells/well (40,000 cells per ml) in the same cell culture condition for the cytotoxicity study. The plates were incubated at 37 °C for 24 h to allow cell to attach. Hydrogels S-90-10-0, S-93-5-2, and S-80-15-5 loaded with and without NGF were added into each well. The plates were incubated at 37 °C for 5 days. The images of PC12 cells were taken by an inverted microscopy (Nikon ECLIPSE TE2000-5) equipped with a digital camera. The PC12 cell outgrowth was assessed by average neurite length extending per cell body, using NIH ImageJ software. At least 40 PC12 cells were examined for neurite extension in each well.

Statistical Analysis

Differences between treatment groups were statistically analyzed using a two-tailed Student's T-test for statistical analyses. A statistically significant difference was reported if $p < 0.05$ or less. Data are reported as mean ± S.D. from at least three separate experiments for the NGF release and hydrogel cytotoxicity studies, and at least 40 PC12 cells for the neurite outgrowth studies.

Results and Discussion

Synthesis and Characterization of Hydrogels

Three sets of thermoresponsive biodegradable hydrogels S-80-15-5, S-90-10-0 and S-93-5-2 are designed and synthesized. The rationale for designing the hydrogels with the three different feeding ratios of the three components PNIPAAm, PLLA and dextran is that PNIPAAm component should have majority amount so that the hydrogels can be thermoresponsive for aqueous loading of NGF with high efficiency at temperature below the LCST; and PNIPAAm and dextran components can modulate the degradation of PLLA component[24,25] and subsequence sustained NGF release profiles at temperature above the LCST. In addition to the three sets of thermoresponsive biodegradable hydrogels, PNIPAAm homopolymer hydrogels without degradable components PLLA and dextran were also synthesized. Figure 1 shows the optical images for hydrogel S-80-15-5 swollen in water at 25 and 37 °C, below and above the LCST, respectively.[25] All the hydrogels in the dry and swollen states are smaller than a nickel coin. PNIPAAm hydrogles are swollen and transparent at 25 °C and become twice smaller in term of diameter (Figure 1) and opaque when temperature is increased to 37 °C due to the thermoresponsive property of the PNIPAAm polymer chains. S-80-15-5 hydrogels are also more swollen and transparent at 25 °C than 37 °C due to the presence of PNIPAAm polymer in the hydrogels. However, the hydrogels S-80-15-5 are more opaque than the PNIPAAm hydrogels at dry state, and swollen states at both 25 and 37 °C due to the presence of the hydrophobic PLLA component. They have similar size as that of the PNIPAAm hydrogels at dry state and swollen state at 25 °C, but bigger size than that of the PNIPAAm hydrogels at swollen state at 37 °C (Figure 1) due to the presence of the hydrophilic dextran component. This result indicates that the hydrogels S-80-15-5 have less thermo-induced volume change than

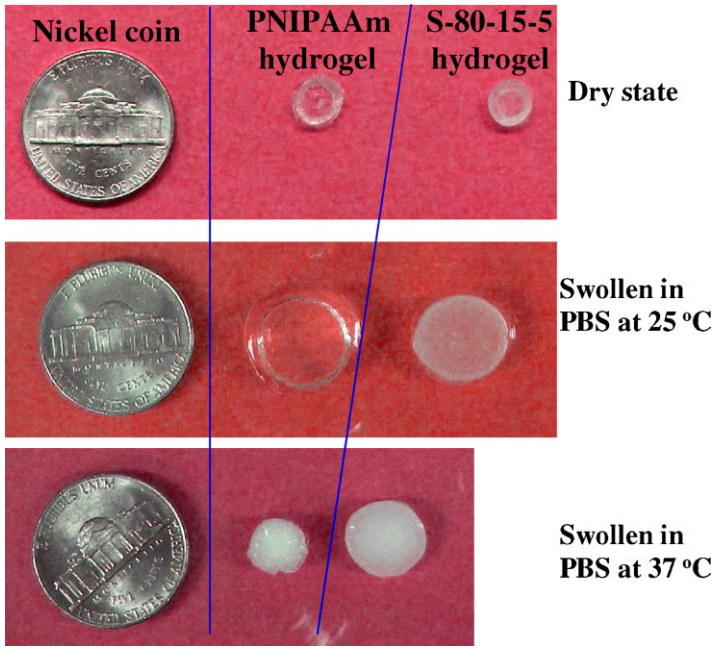


Figure 1.
Opitcal images of S-80-15-5 hydrogels at dry state, and swollen states at 25 and 37 °C.

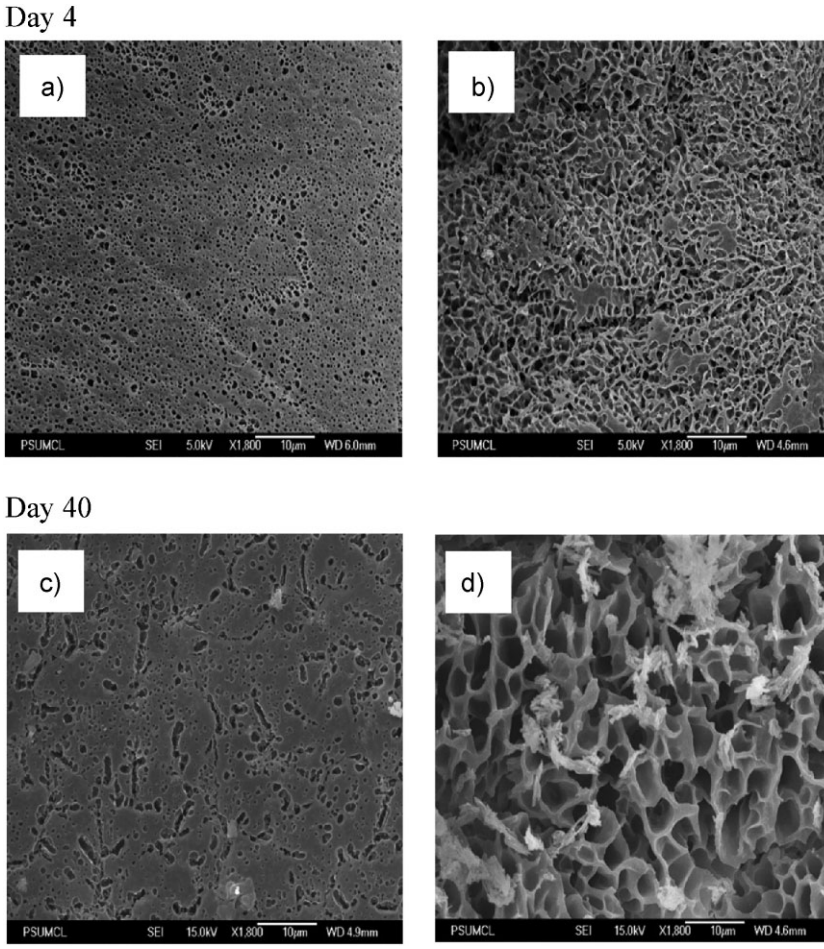


Figure 2.
Surface morphology of hydrogels PNIPAAm (a, c) and S-80-15-5 (b, d) after incubation in PBS (pH 7.4) at 37 °C for 4 (a, b) and 40 days (c, d). The white bars in the pictures represent 10 μm.

the PNIPAAm hydrogels, which is consistent with our previous report.[25]

Figure 2 represents the surface morphology of non-degradable PNIPAAm hydrogels and hydrolytically degradable hydrogels S-80-15-5 after incubation in PBS solution (pH 7.4) at 37 °C for 4 and 40 days. Both the types of hydrogels have smooth surface initially (pictures not shown) at dry state, and show small pores on the surfaces after swelling in water for 4 days, and the pore sizes of the hydrogels S-80-15-5 appear slightly bigger than those of the PNIPAAm hydrogels probably because the S-80-15-5 hydrogels are more heterogeneous than the PNIPAAm hydrogels. The surface morphology of the PNIPAAm hydrogels remains similar from 4 to 40 days except for a minor increase in pore size probably due to very slow PNIPAAm polymer chain relaxation with time. In contrast, S-80-15-5 hydrogels develop much bigger pores and dense polymer aggregate regions on the surface when they continue to be immersed in PBS solvent at 37 °C from 4 to 40 days. The reason is that the PLLA component is continuously degraded by water and subsequently followed by the swelling of the hydrophilic regions and the contrasting of the hydrophobic regions of the hydrogels.

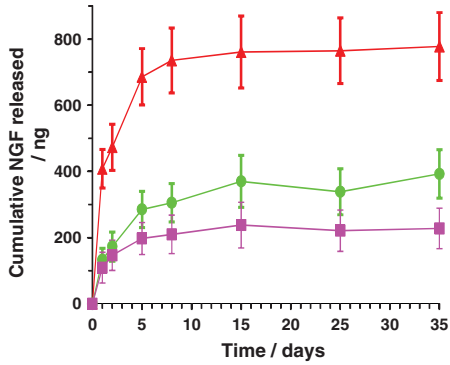


Figure 3.
Cumulative release of NGF from hydrogels S-80-15-5 (▲), S-93-5-2 (●), and S-90-10-0 (■) in PBS (pH 7.4) at 37 °C. Data are reported as mean ± S.D. from at least three separate experiments.

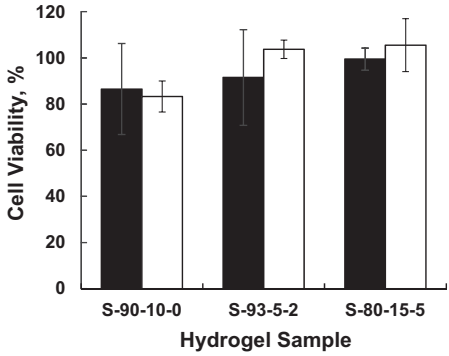


Figure 4.
Effects of hydrogels S-90-10-0, S-93-5-2 and S-80-15-5 on the cell viability of PC12 cells after the hydrogels were incubated with the cells in the cell culture media for 1 (solid bar) and 6 (empty bar) days. Data are reported as mean ± S.D. from at least three separate experiments.

Release Kinetics of NGF from Hydrogels

NGF was loaded into hydrogels S-80-15-5, S-90-10-0 and S-93-5-2 in aqueous solutions at temperature below the LCST of the hydrogels. The loading efficiency of NGF into the hydrogels varied from 8.0 to 27.1% increasing with the feeding amount of the hydrophilic dextran component, such as 8.0% for hydrogels S-90-10-0, 13.7% for hydrogels S-93-5-2, and 27.1% for hydrogels S-80-15-5. The release of NGF from the three types of hydrogels at 37 °C was monitored over a period of 35 days. Figure 3 shows that all the three types of hydrogels can sustain release of NGF for at least up to 15 days due to the degradation of the PLLA component. The amounts of NGF released from the hydrogels at any time point decrease in the order of S-80-15-5 > S-93-5-2 > S-90-10-0. This might be attributed to the fact that the NGF loading amount decrease in the order of S-80-15-5 > S-93-5-2 > S-90-10-0. Other possibilities include that hydrogels S-80-15-5 have more hydrophilic component dextran than hydrogels S-93-5-2 and hydrogels S-90-10-0 have no dextran, and more amount of hydrophilic dextran might facilitate more amount of NGF release from the hydrogels.

Cytotoxicity Studies of Hydrogels

For any new biomaterials, it is important to know their toxicity profiles.[3] In this study, we used MTT assay to evaluate the cytotoxicity of the designed hydrogels (Figure 4). The basic principal underlying MTT assay is that metabolic dysfunction in the cells caused by toxic materials leads to decreased activity of enzymes, which in turn reduces the transformation of MTT to formazan in mitochondria/endosomes. Figure 5 shows the cell viability of PC12 cells after incubated with hydrogels S-90-10-0, S-93-5-2 or S-80-15-5 for 1 or 6 days. All the cell viability was above 85%, indicating that all the designed hydrogels were not toxic to the PC12 cells at the conditions studied.

Biological Activity of NGF Released from the Hydrogels

It is well-known that in the presence of NGF, PC12 cells, a neuron-like cell line, stop their proliferation and differentiate into a neural phenotype by having neurite outgrowth.[14] Therefore, in this study PC12 cells were used as neuronal model cells to evaluate the biological activity of NGF released from the hydrogels S-90-10-0, S-93-5-2 or S-80-15-5 incubated in the cell culture media for 5 days. From the pictures in Figure 5, it can be seen in the presence of the NGF-loaded hydrogels S-80-15-5, PC12 cells start to grow small short neurites after

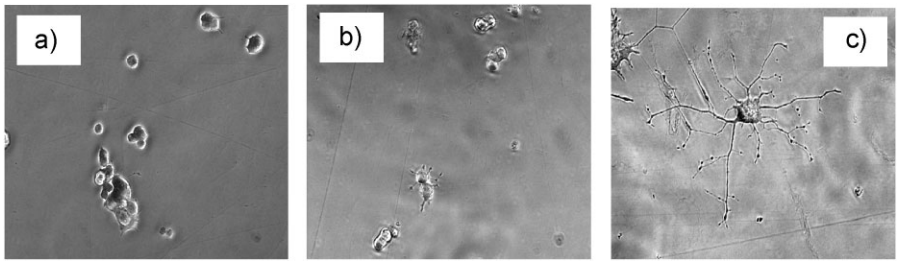


Figure 5.
Neurite outgrowth of PC12 cells after treatment with NGF-loaded S-80-15-5 hydrtogels at 37 °C for 1 (a), 2 (b), and 5 days (c), recorded by inverted microscopy.

1 day treatment. The number of the cells with neurites and the length of the outgrowth neurites increase with the treatment time from 1 to 2 and then to 5 days. It is worth to mention that PC12 cells treated with hydrogels alone without NGF show no noticeable neurite outgrowth. The effects of all three types of NGF-loaded hydrogels on the neurite outgrowth were quantified after PC12 cells were treated with the hydrogels for 5 days. Figure 6 shows that PC12 neurite lengths per cell body increase in the order of S-90-10-0 < S-93-5-2 < S-80-15-5. The reason may be due to the fact that the hydrogels increase the NGF release amounts in the order of S-90-10-0 < S-93-5-2 < S-80-15-5, and within the concentration range approximately a few ng/ml to a couple of hundreds ng/ml of the NGF released in the media, the higher amount is the NGF released, the longer outgrowths the neurite.

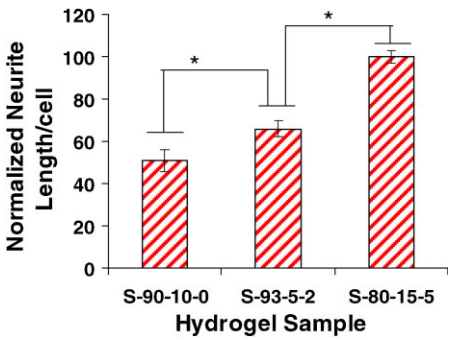


Figure 6.
Neurite outgrowth lengths per single cell body after PC12 cells were treated with NGF-loaded hydrogels S-90-10-0 and S-93-5-2, and S-80-15-5 in the cell culture media at 37 °C for 5 days. The lengths were measured by NIH ImageJ software, and normalized by setting the average length resulting from the presence of NGF-loaded hydrogels S-80-15-5 as 100. At least 40 PC 12 cells were examined for neurite extension in each well. * $p < 0.05$.

Conclusion

Novel thermo-responsive and biodegradable hydrogels composed of N-isopropylacrylamide (NIPAAm) as a thermoresponsive unit, poly(L-lactic acid) (PLLA) as a hydrolytically degradable and hydrophobic unit, and dextran as an enzymatically degradable and hydrophilic unit, have been synthesized and characterized for sustained release of NGF. The hydrogels generate bigger pores along degradation in PBS (pH 7.4) solution at 37 °C with time. The composition of the hydrogels can be tailored to achieve different NGF loading amount in aqueous solution and different NGF release kinetics. The loading efficiency can be as high as at least 27%. The synthesized hydrogels are not toxic to neuron-like PC12 cells, and sustain release of NGF for at least 15 days. The released NGF has biological activity by promoting neurite outgrowth of PC12 cells, and generates different neurite length per cell body depending on the composition of the hydrogels and subsequent different amount of NGF release in the cell culture media at 37 °C. The presence of small amount of dextran component and the balanced amount of the dextran and the degradable

PLLA components in the hydrogels play important roles in achieving high NGF loading efficiency and long neurite outgrowth. In summary, the developed thermo-responsive and biodegradable hydrogels have great potential to be used for controlled release of NGF for the treatment of neurological disorders.

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