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Nanogel tectonic porous gel loading biologics, nanocarriers, and cells for advanced scaffold

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ABSTRACT

We developed a new self-assembled amphiphilic nanogel-crosslinked porous (NanoCliP) gel that can trap proteins, liposomes, and cells. The NanoCliP gel was prepared by Michael addition of a self-assembled nanogel of acryloyl group-modified cholesterol-bearing pullulan to pentaerythritol tetra (mercaptoethyl) polyoxyethylene, followed by freezing-induced phase separation. Dynamic rheological analysis revealed that the storage modulus (G') of the NanoCliP gel was approximately 10 times greater than that of a nonporous nanogel-crosslinked gel. Two-photon excitation deep imaging revealed that the NanoCliP gel comprises interconnected pores of several hundred micrometers in diameter. The NanoCliP gel trapped proteins and liposomes via hydrophobic interactions because its amphiphilic nanogels exhibit chaperone-like activity. Mouse embryonic fibroblasts penetrated the interconnected pores and adhered to the porous surface of fibronectin-complexed NanoCliP gel. *In vivo*, the NanoCliP gel enhanced cell infiltration, tissue ingrowth, and neovascularization without requiring exogenous growth factors, suggesting that the NanoCliP gel is a promising scaffold for tissue engineering.

1. Introduction

Polymer gels are often used as soft functional materials in biotechnological and biomedical applications [1]. For example, hydrogels are frequently used as scaffolds for tissue engineering because of their hydrated, three-dimensional (3D) structures that mimic soft biological tissues, high biocompatibility, minimal inflammatory responses, and low toxicity [2,3]. Biological activities during tissue regeneration are highly dependent on the 3D structure of the scaffold [4]. The interconnected porous structure allows rapid ingrowth of cells, promotes vascularization, and enhances nutrient diffusion or waste exchange to support successful tissue regeneration [5,6]. Porous scaffolds also provide temporary spaces and mechanical support for cells to grow, facilitating tissue regeneration [7]. Accordingly, there is a need for hydrogel scaffolds with pores of several hundred micrometers in diameter, which are large enough to allow cell ingrowth. However, the polymer network of hydrogels is generally much smaller than cells, which delays cell migration, vascularization, and tissue invasion [8]. Therefore, the design of a hydrogel scaffold with a well-controlled, microscale porous structure is a major challenge.

Recently, several strategies that combine therapeutic molecules (e.g., proteins, peptides, and growth factors) with hydrogels have been investigated to improve the performance of the scaffold. However, some of these strategies were unfavorable because proteins are often unstable and rapidly denature or degrade in the body. Therefore, novel methods are needed to handle therapeutic molecules based on hydrogels.

To address these issues in the development of hydrogel scaffolds for tissue regeneration, we investigated a novel strategy for fabricating a nanogel-based hydrogel scaffold with well-controlled interconnected pores that could control the release of therapeutic molecules. We first reported the development of physically cross-linked nanogels by self-assembly of associating polymers, such as amphiphilic polysaccharide. Self-assembled polysaccharide nanogels are useful components of protein carriers such as cancer and nasal vaccines [9].

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More recently, a new method, termed nanogel tectonics, was proposed to construct hydrogel materials with a hierarchic structure. The nanogel-crosslinked (NanoClik) gel was synthesized using self-assembled nanogels as the building blocks [10]. Nanogels are used as individual components for building nano-integrated functional hydrogel systems. The NanoClik gel consists of a physically crosslinked network formed by self-assembly of amphiphilic polysaccharide and a chemically crosslinked network between the nanogels. The self-assembled nanogel displays chaperone-like activity (trapping proteins by hydrophobic interactions, stabilizing them, and releasing them in their native form). The chaperone activity of these nanogels is also maintained after gelation. It is possible to control the degradation and release of nanogels by the type of chemical bond responsible for the chemical crosslinks. The NanoClik gel has been used as a scaffold for tissue engineering, including bone regeneration. However, the extent of cell migration, vascularization, and tissue invasion inside the gel is limited because the gel's polymer network is very small. In this study, we developed a new hierarchic structural hydrogel with a macroporous structure using the nanogel tectonic principle. The nanogel-crosslinked porous (NanoCliP) gel with macro-sized pores of several hundred micrometers was prepared by Michael addition of acryloyl group-modified cholesterol-bearing pullulan (CHPOA) to pentaerythritol tetra (mercaptoethyl) polyoxyethylene (PEGSH), followed by freezing-induced phase separation. We systematically examined the structure, mechanical properties, degradation behavior, and cellular interaction of the NanoCliP gel. Finally, we subcutaneously transplanted the NanoCliP gel into mice and examined cell infiltration and neovascularization.

2. Materials and methods

2.1. Chemicals

CHP, in which pullulan (molar weight = 1×10^5 g/mol) was substituted with 1.2 cholesterol moieties per 100 anhydrous glucoside units and PEGSH (molar weight = 1×10^4 g/mol) were purchased from NOF corporation (Tokyo, Japan). 2-Acryloyloxyethyl isocyanate (AOI) was purchased from Showa Denko K. K (Tokyo, Japan). Di-*n*-butyltin (IV) dilaurate (DBTDL, 95%), Rhodamine B isothiocyanate and FITC–insulin from the bovine pancreas were purchased from Sigma–Aldrich Co., LLC (St. Louis, MO, USA). Dehydrated dimethylsulfoxide (DMSO) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Life Technologies Corporation (Carlsbad, CA, USA). All other regents were obtained from commercial sources and were used without further purification.

2.2. Synthesis of rhodamine-labeled CHP (CHP-Rh)

CHP-Rh was synthesized as previously described [11]. Briefly, CHP (3.0 g) was dissolved in 150 ml of dehydrated DMSO containing DBTDL (20 mM). Separately, rhodamine B isothiocyanate (4 mM) was dissolved in dehydrated DMSO and stirred for 30 min at room temperature. The two solutions were combined and stirred at 45 °C for 24 h and added to excess ether/ethanol. The precipitates were dissolved in DMSO, dialyzed with DMSO for 1 day and milli-Q water for 7 days, and then lyophilized. The degree of substitution (DS) of rhodamine groups was assessed by ultraviolet (UV) measurement at 558 nm using a UV/visual spectrophotometer (V-660; Jasco, Tokyo, Japan). CHP-Rh contained 0.3 rhodamine groups per 100 anhydrous glucoside units.

2.3. Synthesis of acryloyl group-modified CHP-Rh (CHPOA-Rh)

CHPOA-Rh was synthesized as previously described [11]. Briefly, CHP-Rh (2.5 g) was dissolved in 100 mL of dry DMSO, followed by the addition of DBTDL (10 mM) and AOI (30 mM). The mixture was stirred at 45 °C for 24 h and added to excess ether/ethanol. The precipitates were dissolved in DMSO, dialyzed with milli-Q water, and lyophilized. The DS of acryloyl groups was determined by ^{1}H nuclear magnetic resonance spectroscopy (400 MHz, Avance 400; Bruker BioSpin K.K., Kanagawa, Japan, DMSO-d6/D2O = 9/1 (v/v), 25 °C, δ = 0.64 (s, 3H, cholesterol 18-H), 0.80–2.40 (m, cholesterol H), 2.95–4.00 (glucose, 2-H, 3-H, 4-H, 5-H and 6-H), 4.69 (1H, glucose 1-H (1 → 6)), 5.03 (2H, glucose, 1-H (1 → 4)), 5.96–6.37 (3H, CH_2=CH–). The DS was 24.1 acryloyl groups per 100 anhydrous glucoside units.

2.4. Preparation of the NanoCliP gel

The NanoCliP gel was prepared by Michael addition of the acryloyl groups of CHPOA-Rh to the thiol groups of PEGSH. Briefly, CHPOA-Rh was dissolved in PBS (pH 7.4). Separately, PEGSH was dissolved in PBS and added to the CHPOA-Rh solution. The mixture was dropped between polytetrafluoroethylene membranes and kept at 37 °C for 1 h to obtain a disk-shaped nanogel-crosslinked hydrogel. The molar ratio of acryloyl groups to thiol groups was 2:1. The NanoClik gel was then transferred to a polystyrene dish and was frozen at −28 °C for 30 min, and thawed at 25 °C. As a comparison, PCliP gel was prepared in the same manner.

2.5. Two-photon laser scanning microscopy (TP-LSM)

TP-LSM of the rhodamine-labeled NanoCliP gel was conducted using a multi-photon microscope (LSM780; Carl Zeiss, Oberkochen, Germany) equipped with a Coherent Chameleon Vision S laser source and GaAsP detector. Images of the rhodamine-labeled NanoCliP gel were obtained at a magnification of 25× with an excitation wavelength of 820 nm. A 3D image was reconstructed from a sequence of 200 fluorescent images (1.3 μm thick) acquired vertically with the z-stack function using Zen software (Carl Zeiss).

2.6. Freeze-fracture transmission electron microscopy (FF-TEM)

The NanoClik and NanoCliP gels (φ = 4 mm, thickness = 1.5 mm) were immersed in 30% glycerol in PBS for 1 week at 20 °C. The specimens were frozen in slush nitrogen and fractured at −130 °C under a vacuum using a freeze-fracture apparatus (JFD-V; JEOL Ltd., Tokyo, Japan). The fractured surface was replicated with platinum–carbon. The replicas were washed with deionized water, mounted on copper grids, and then observed by TEM at an accelerating voltage of 100 kV (JEM-1010; JEOL Ltd.).

2.7. Dynamic rheological analysis

The rheological measurements were conducted in the dynamic oscillation mode using a dynamic shear rheometer (MCR302; Anton Paar GmbH, Graz, Austria) with 25-mm-diameter stainless steel parallel plates. The sample temperature was controlled using a hood-type chamber equipped with two Peltier units at the top and the bottom of the chamber. The NanoCliP gel was prepared on the Peltier stage of the rheometer as follows. First, 600 μl of the mixture of CHPOA and PEGSH was quickly dropped onto the rheometer stage, which was pre-set at 20 °C. The parallel plate was then lowered until the gap between the plates was 1 mm and the temperature was quickly increased to 37 °C. The storage modulus (G') and loss modulus (G'') were measured to monitor gelation at an angular frequency of 2π rad/s and strain of 0.5% as a function of time until an apparent equilibrium modulus was observed. To obtain the NanoCliP gel, the NanoClik gel was frozen in a stress-controlled mode on the rheometer stage at −25 °C for 30 min, and then thawed at 25 °C. The change in viscoelastic modulus over time was measured at an angular frequency of 2π rad/s and strain of 0.5% for 250 s. Frequency sweep measurements were also conducted in which the angular frequency ranged from 10^{-1}–10^2 rad/s at a constant strain of 0.5%.

2.8. Protein trapping and release experiments

The NanoCliP gel (φ = 6 mm, thickness = 1 mm) was immersed in 2 ml of FITC–insulin (50 μg/ml) in PBS at 25 °C. Then, 50 μl samples of the FITC–insulin solution were obtained at specific times and the fluorescence intensity of each sample was measured using a fluorescent spectrometer (FP-8500; JASCO, Tokyo, Japan) at an excitation wavelength of 495 nm and an emission wavelength of 519 nm. The encapsulation efficiency was estimated based on the decrease in fluorescent intensity of FITC–insulin in PBS.

The release of FITC–insulin from the NanoCliP gel in the presence of serum was also measured. The rhodamine-labeled NanoCliP gel complexed with FITC–insulin (φ = 6 mm, thickness = 1 mm) was immersed in 1 ml of PBS supplemented with 10% FBS at 37 °C. Then, 5 μl samples of the supernatant were collected at specific times and their fluorescence intensity was measured using a fluorescent spectrometer equipped with a one-drop measurement unit (SAF-851; JASCO). The experiments were repeated three times and the results are expressed as the mean ± standard deviation (SD).

2.9. Gel degradation experiments

The rhodamine-labeled NanoCliP gel (φ = 6 mm, thickness = 1 mm) was immersed in 4 ml of PBS (pH 6.02, 7.36 or 8.01) at 37 °C. Then, 100 μl samples of PBS were collected at specific times. After collecting each sample, 100 μl of fresh PBS was immediately added to maintain a fixed volume. The cumulative release of nanogels from the NanoCliP gel was estimated by fluorescent spectrometry at an excitation wavelength of 550 nm and an emission wavelength of 580 nm.

We also investigated the degradation of the NanoCliP gel in the presence of serum. The rhodamine-labeled NanoCliP gel (φ = 6 mm, thickness = 1 mm) was immersed in 1 ml of PBS supplemented with 10% FBS at 37 °C. Then, 5 μl samples of the supernatant were collected at specific times and the fluorescence intensity of each sample was measured using a fluorescent spectrometer equipped with a one-drop measurement unit. The experiments were repeated four times, and the results are expressed as the mean ± SD.