Injectable, photoresponsive hydrogels for delivering neuroprotective proteins enabled by metal-directed protein assembly

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INTRODUCTION

Over years, new methods for protein conjugation and assembly have opened a venue to diverse bioactive protein hydrogels (1, 2). These protein-based materials, noted for their genetic programmability and functional diversity, have emerged as a promising alternative to those derived from other natural or synthetic polymers (3, 4). For instance, the use of genetically encoded click chemistry such as SpyTag/SpyCatcher or SnoopTag/SnoopCatcher, a peptide/protein pair that can covalently stitch together protein molecules by forming an isopeptide bond (5, 6), has led to the creation of various hydrogels and “living” soft materials (7–19). Compared with those efforts of creating hydrogels from synthetic polymers or natural precursors, this approach toward hydrogel design is advantageous in that it circumvents the need for chemical modification and renders better cytotoxic or biocompatibility. Nevertheless, to form bioactive hydrogels, those protein building blocks have to be judiciously designed to have multiple reactive domains in place, which can hardly be generalized insofar as easy fabrication and biofunctionalization are concerned.

Metal-ligand coordination interactions are crucial for a wide range of biological phenomena such as transcriptional regulation, protein phase transition, and underwater adhesion (20). One noted example is mussel foot proteins that can undergo metal-induced phase transition and self-assemble into self-healing fiber-like materials, an underlying mechanism by which mussels bind to underwater substrates (21). This unique feature has inspired the creation of several metal-coordinated polymeric materials with exceptional mechanics, such as extremely tough hydrogels and self-healing solid materials (22–25). The combined use of multiple, kinetically distinct metal (Ni2+, Cu2+, and Zn2+)–ligand interactions has enabled the assembly of four-arm histidine-modified poly(ethylene glycol) (4A-PEG-His) into viscoelastic hydrogels with decoupled mechanics and spatial structure (26). Furthermore, cross-linking 4A-PEG-His or His6-tagged proteins with cobalt ions has led to the formation of redox-responsive molecular networks that transitioned from viscoelastic liquids to elastic solids upon oxidation of Co2+ to Co3+ (27, 28).

Recombinant proteins have often been fused with a polyhistidine-tag (His6-tag) for the ease of purification by the immobilized metal-ion affinity chromatography (29). We envisioned that the coordination interactions between His6-tag and transition metal ions such as Co, Ni, Cu, and Zn might also provide a general approach for assembling recombinant proteins into higher-order structures. In this study, we leveraged the metal/His6-tag interactions, in combination with adenosylcobalamin (AdoB12)–induced oligomerization, to assemble the photoreceptor His6-CarH2 proteins into a macroscopic photoresponsive hydrogel system, which—the Zn2+-coordinated hydrogel in particular—allowed for encapsulation and release of cells and proteins in a light-dependent manner. Moreover, these materials are injectable and proved to be effective in delivering neuroprotective cytokines like leukemia inhibitory factor (LIF) into injured mouse optic nerves, showing their potential in therapeutic delivery and neuroregeneration.

RESULTS AND DISCUSSION

Protein construct design
To examine the feasibility of using metal ions to assemble His6-tagged recombinant proteins into a hydrogel while preserving their molecular function, we chose as a model system the His6-tagged...
recombinant protein, SpyTag-ELP-CarH_C-ELP-SpyTag (ACA) (Fig. 1 and fig. S1), which has previously exhibited marked solubility and expression yield in *Escherichia coli* (11). The domain central to this construct, CarH_C, is a B_{12}-dependent photoreceptor derived from a bacterial transcriptional regulator that controls the biosynthesis of the carotenoid pigments (30–32). The C-Co bond within AdoB_{12} is sensitive to green light (522 nm). CarH_C self-assembles into tetramers upon binding to the cofactor AdoB_{12} in the dark and disassembles into monomers on light exposure, accompanied by the cleavage of the photolabile C-Co bond within AdoB_{12}, the release of 4′,5′-anhydroadenosine, and the coordination of His^{132} to the Co center (Fig. 1). The flanking ELP domains consist of repeating pentapeptides (VPGXG)_{15}, where X represents either Val or Glu at a 4:1 ratio, a composition that lead to a lower critical solution temperature (LCST) of ~45° to 60°C (table S1) (33). Since all experiments in this study were performed at either 25° or 37°C, far below this LCST, the thermally induced phase transition of these ELP domains is negligible. In addition, the presence of the SpyTag domains, while not the focus of this study, does offer the possibility of covalently decorating the materials via SpyTag/SpyCatcher chemistry. To assess the role of His6-tag, we also constructed a His6-tagged ACA protein, of which the His6-tag can be cleaved by tobacco etch virus (TEV) protease.

**Metal-directed protein assembly**

His6-tag binds tightly to divalent transition metal ions such as Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} (29, 34). We first tested Co^{2+}, with the molar ratio of metal to ACA varied from 1:6 to 8:1, to determine the proper stoichiometry needed to effectively assemble His6-tagged ACA. It turned out that mixing ACA [2.4 mM, 10 weight % (wt %)] with Co^{2+} (4.8 mM) at a 1:2 molar ratio, followed by addition of AdoB_{12} (2.4 mM), led to the formation of a solid-like material. On the contrary, the use of insufficient amounts of metal ions such as an equimolar ratio of ACA to Co^{2+} failed to initiate the gelation of ACA in the presence of AdoB_{12}, while excess metal ions (Co^{2+}:ACA ≥ 4:1) resulted in insoluble protein aggregates. Similarly, the other transition metal ions including Ni^{2+}, Cu^{2+}, and Zn^{2+} also exhibited the ability to induce the gelation of ACA at the 1:2 molar ratio (ACA:M) in the presence of AdoB_{12}. Note that the ACA protein, after removal of His6-tag by TEV proteolysis, failed to gel in the presence of divalent metal ions and AdoB_{12}, showing that the His6-tag/metal coordination is essential for the observed hydrogel formation, whereas the possible nonspecific interactions between metal ions and the other residues of ACA played negligible roles in gelation (fig. S2).

Rheological measurements in the time sweep mode revealed the gelation kinetics of the ACA/M^{2+} complex upon addition of AdoB_{12} in the dark, of which the storage modulus $G'$, substantially larger than the loss modulus $G''$, reached plateau within 15 min (Fig. 2A). Frequency sweep tests on all four types of M^{2+}-coordinated gels revealed $G' \sim 1$ kPa and $G'' \sim 0.1$ kPa over the frequency range of 0.01 to 100 rad/s, confirming the formation of solid materials (Fig. 2B). Only the Cu^{2+}-coordinated gel, but not the others, displayed $G'$ and $G''$ highly dependent on shear frequency; a local maximum of $G''$ appeared at the frequency of 0.03 rad/s, pointing to the viscoelastic nature of this material (Fig. 2B). The affinity of the metal ions toward electron-donating groups typically follows the order of...
Cu\(^{2+}\) > Ni\(^{2+}\) > Zn\(^{2+}\) ≈ Co\(^{2+}\), whereas their selectivity toward a particular ligand is opposite (Cu\(^{2+}\) < Ni\(^{2+}\) < Zn\(^{2+}\) ≈ Co\(^{2+}\)) (35, 36). The appearance of this local maximum in the G\(^{″}\), as well as relatively higher final modulus G\(^{′}\) (table S2), may reflect the contributions from additional dynamic, nonspecific binding of Cu\(^{2+}\), with the lowest selectivity, to the side chains of other residues other than the His6-tag.

Immersing the gels with tris-buffered saline (TBS) in the dark led to less than 10% protein loss after 5 days, showing the marked stability of these metal-coordinated protein networks (Fig. 2C). Meanwhile, these M\(^{2+}\)-coordinated gels underwent a rapid gel-sol transition, accompanied by markedly decreased G\(^{′}\), under continuous white light-emitting diode (LED) illumination (35 klux), an intensity comparable to that of outdoor sunlight (~10 to 100 klux) according to the National Optical Astronomy Observatory in the United States (Fig. 2D and table S2) (37). This light-induced phase transition can be attributed to the photolysis of AdoB12 and the disassembly of tetrameric CarH C within the protein networks (Fig. 1). Pulsed illumination, on the other hand, provided an effective means to modulate the gel mechanics (fig. S3). On brief light exposure, the material, instead of undergoing a complete gel-sol transition, remained as a solid while exhibiting somewhat decreased G\(^{′}\) (fig. S3). Together, these results demonstrated the feasibility of converting His6-tagged CarHC into stable, photoresponsive hydrogels by tapping into the His6-tag/M\(^{2+}\) coordination interactions.

**Redox-responsive cobalt-coordinated protein networks**

The Co\(^{2+}\) complexes are thermodynamically unstable and kinetically labile, which is in stark contrast to the Co\(^{3+}\) complexes that are thermodynamically stable and markedly slow in ligand exchange. This contrast, which is unique to Co\(^{2+}\)/Co\(^{3+}\), has recently been used to create hydrogels with marked redox responsiveness (27, 28). Time sweep tests on the Co\(^{3+}\)/ACA complex, which was generated by treating the Co\(^{2+}\)/ACA complex with H\(_2\)O\(_2\) (fig. S4). The cofactor is essential for the gelation, as the mixture of ACA and Co\(^{3+}\) in the absence of AdoB12 failed to gel (fig. S4). The Co\(^{3+}\)-coordinated gel, with G\(^{′}\) increased from ~0.6 to ~1.0 kPa and slightly stiffer than the Co\(^{2+}\) gel (fig. S5A), turned out to be more stable; immersing these Co\(^{3+}\)-coordinated gels into TBS led to little erosion (<5%), while the gels were completely dissolved in the presence of ascorbate (10 and 50 mM) within a few hours, suggesting that the reduction of Co\(^{3+}\) to Co\(^{2+}\) indeed destabilized the gel networks (fig. S5B). This redox responsiveness of the Co-coordinated CarHC gel presents an additional means to modulate the material properties, which is orthogonal to its photoresponsiveness and can be handy for the applications involving complex biological systems.

**Self-healing and injectability**

Strain sweep tests revealed linear viscoelastic regions with varied linearity limits that were dependent on the metal ions (Fig. 3, A to D). The gels containing Co, Ni, Cu, and Zn displayed the linearity limits (yield points) at γ\(_y\) ~ 100, 70, 50, and 141%, respectively, and the flow points, where the G\(^{′}\) and G\(^{″}\) curves cross over, at γ\(_f\) ~ 158, 177, 100, and 250%, respectively (Fig. 3, A to D). The G\(^{′}\) curves of the Co, Cu, and Zn gels showed steep downturns at the corresponding yield points, accompanied by the sharp rises of the G\(^{″}\) curves, strongly suggesting brittle fracturing behavior. According to their flow index.

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**Fig. 2. Physical properties of metal-coordinated protein networks.** (A) Evolution of G\(^{′}\) and G\(^{″}\) of the CarH C protein complexed with divalent transition metal ions (Co\(^{2+}\), Ni\(^{2+}\), Cu\(^{2+}\), and Zn\(^{2+}\)) in the presence of AdoB12 at room temperature. (B) Frequency sweep tests on these CarH C hydrogels (10 wt %) at room temperature in the dark. The strain was fixed at 5%. (C) Erosion profiles of the CarH C hydrogels (60 µl) immersed in 1 ml of TBS at room temperature in the dark. Data are presented as means ± SD (number of gels, n = 3). (D) Light-induced gel-sol transition. On exposure to white LED light (35 klux), G\(^{′}\) and G\(^{″}\) of the gels were monitored at a fixed shear frequency (5 rad/s) and strain (5%). Photo credit: Bojing Jiang, Hong Kong University of Science and Technology.
values ($\gamma_f/\gamma_y$), the tendency of these gels to brittle fracturing follows this order: Co ($\gamma_f/\gamma_y \sim 1.58$) > Zn ($\gamma_f/\gamma_y \sim 1.77$) > Cu ($\gamma_f/\gamma_y \sim 2.00$) > Ni ($\gamma_f/\gamma_y \sim 2.58$). The yielding behavior is deemed to be important for injectable delivery of live cells because the sharp yielding transition observed from the hydrogels may allow encapsulated cells to survive the injection process (38).

Continuous step-strain measurements showed the quick recovery of these hydrogels after network failure. The hydrogel networks were ruptured by applying a high strain (250%) for 500 s, which led to a liquid-like status ($G'' > G'$). Switching to a low-amplitude strain (5%) restored the solid-like mechanics ($G' > G''$) immediately; the $G'$s could be restored even after 6 cycles of network rupture (Fig. 3, E to H). The robust self-healing behavior reflects the contributions from the reversible metal/His6-tag coordination interactions within the protein networks. The robust self-healing behavior of these metal-coordinated gels, together with their sharp yielding transition, is also highly indicative of injectability. The Zn$^{2+}$-coordinated gels exhibited marked injectability, which points to their potential for less invasive therapeutic delivery (movie S1).

Cell encapsulation and light-induced release
The material system that enables facile encapsulation and recovery of cells is highly desirable for basic research and cell-based therapies (11, 39, 40). The inducible phase transition behavior of the $M^{2+}$/ACA complexes provides the possibility for controllable cell encapsulation and release. Given that Zn$^{2+}$, noted for its critical roles in cell signaling and regulation, is relatively abundant in biological systems (41), it is conceivable that the Zn$^{2+}$-coordinated gels could be better suited for cell encapsulation or in vivo applications than the other metal-coordinated ones. In view of this, the

Fig. 3. Strain sweep tests on metal-coordinated CarH$_2$ hydrogels. (A to D) Strain sweep tests showing the yield points ($\gamma_y$) and flow points ($\gamma_f$) at high-amplitude strain. (E to H) Continuous step-strain measurements. Low (5%) and high strains (250%) were used alternately.
rest of this study has been largely focused on the feasibility of using the Zn\textsuperscript{2+}-coordinated gels for cell-based and in vivo applications.

We first examined their ability to encapsulate mouse 3T3 fibroblasts and human mesenchymal stem cells (hMSCs). The cells were suspended in the ACA/Zn\textsuperscript{2+} (1:2) solution, followed by addition of AdoB\textsubscript{12} to initiate gelation. After 45 min, the gels were immersed in culture medium and incubated in the dark (37°C, 5% CO\textsubscript{2}) for 12 hours. Standard live/dead staining assays were performed to determine the cell viability. It turned out that the majority of fibroblasts (90.0 ± 3.6%) and MSCs (88.0 ± 1.4%) remained viable after 12-hour encapsulation (Fig. 4), confirming the cytocompatibility of these Zn\textsuperscript{2+}-coordinated gels.

On light exposure, the encapsulated cells can be readily released from the Zn\textsuperscript{2+} gels within 8 min, accompanied by a gel-sol transition (Fig. 4, fig. S6, and movies S2 and S3). These metal-coordinated photoresponsive hydrogels, with low toxicity and capable of easy cell encapsulation and recovery, may represent a versatile system for three-dimensional (3D) cell culturing and cell transplantation.

**Immobilization and release of His6-tagged proteins**

As protein drugs often exhibit a brief half-life time in vivo, controllable delivery/release offers a possible way to improve their efficacy (42, 43). We envisioned that the His6-tagged proteins could be readily immobilized onto the hydrogel networks via the metal coordination and then could be released via the light-induced gel-sol transition. To test this, we chose His6-tagged green fluorescent protein (GFP) as a model substrate. It turned out that only scarce amounts of GFP (<10%) were released from the gels decorated with His6-GFP in TBS in the dark; the gels remained largely intact (fig. S7). On the other hand, those Co\textsuperscript{3+} and Zn\textsuperscript{2+} gels, which were exposed to light, were fully dissolved in TBS within 50 and 100 min, respectively, both accompanied by a complete release of His6-GFP (fig. S7). These results have proven the feasibility of using these metal-coordinated photoresponsive hydrogels for optically controlled delivery of protein therapeutics.

**LIF-laden hydrogels for sustained signal transducers and activators of transcription 3 activation**

Previous studies have established that LIF is neuroprotective and facilitates axon growth (44, 45). It has also been shown that injection of this cytokine into lesion sites led to enhanced neurite growth, albeit moderately (46). The stiffness of neural tissue ranges from ~150 Pa close to the cortical plate in a human brain (47) to ~1500 Pa near its apical surface (48), comparable to the G’ of the Zn\textsuperscript{2+}-coordinated CarH\textsubscript{2} gels (~1 kPa). Given that they also exhibited several important features including injectability, cytocompatibility, and inducible phase transitions, we envisioned that these materials could be used for the controlled and sustained delivery of LIF into nervous systems. We first examined whether these gels could prolong the LIF signaling in vivo, which can be gauged by the level of Tyr\textsuperscript{705} phosphorylation in signal transducers and activators of transcription 3 (STAT3), a transcription factor that is important for neuroprotection and repair (49). C57BL/6 mice received intravitreal injection of TBS, LIF solution in TBS, LIF-free (Ctrl) gels, or LIF-laden gels. They were then kept under the dark or normal circadian conditions to examine the possible influence of protein release rates on the STAT3 activation in vivo. The population of p-STAT3–positive retinal ganglion cells (RGCs) in the retinas, which is indicative of LIF signaling, was analyzed and quantified using immunostaining several days (1, 5, 9, or 14 days) after the administration. It turned out that injection of the blank vehicle (TBS) or the LIF-free gels only led to a rather low level of p-STAT3 in RGCs after 1 day, while injection of either the LIF solutions or the LIF-laden gels immediately enhanced the phosphorylation of STAT3 on day 1, reflecting the activity of LIF in both formulations in vivo. The level of STAT3 phosphorylation remained high in the optic nerves that received the LIF-laden gels under both dark and normal circadian conditions after 5 days but not in those with the LIF solutions; approximately 60% p-STAT3–positive RGCs were observed 5 days after the injection of the LIF-laden gels, regardless of the light conditions (Fig. 5).

The influence of light on the LIF delivery in vivo became noticeable in the prolonged studies (9 and 14 days after the administration).

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**Fig. 4. Light-induced release of encapsulated cells.** (A and C) Mouse 3T3 fibroblasts and hMSCs were encapsulated by Zn\textsuperscript{2+}-coordinated CarH\textsubscript{2} hydrogels in the presence of AdoB\textsubscript{12} (red) and cultured for 12 hours, followed by live/dead staining in situ. The release of cells was initiated by quickly switching to the epifluorescence mode using a blue-light filter set (480 nm, ~1.35 W/cm\textsuperscript{2}). Bright-field images show the gel boundaries (dash lines). (B and D) Recovered 3T3 fibroblasts (B) and hMSCs (D) exhibited marked viability (90.0 ± 3.6% and 88.0 ± 1.4%, respectively). Data are presented as means ± SD (n = 3). Representative fluorescence micrographs are shown. PBS, phosphate-buffered saline.
On day 9, nearly all p-STAT3–positive RGCs disappeared in the mice that received the LIF-laden gels under normal circadian conditions, while approximately 25 and 10% p-STAT3–positive RGCs remained in the mice reared in the dark on days 9 and 14, respectively, showing the marked ability of these materials for sustained, photoresponsive delivery of LIF in vivo (Fig. 5B). Together, these results proved the feasibility of using the metal-coordinated protein hydrogels to achieve prolonged biological signaling in vivo.

**LIF-laden hydrogels promote axon regeneration**

We further examined the influence of these LIF-laden hydrogels on neuroprotection and axon regeneration using an optic nerve crush model. The C57BL/6 mice, after optic nerve crush, were injected intravitreally with the vehicle (TBS), the LIF solutions in TBS, or LIF-laden gels. While the injection of the LIF solutions exhibited little effect on the RGC survival in the lesion sites 2 weeks after optic nerve crush, the administration of the LIF-laden gels led to ~30% increase in the population of live RGCs under both dark and normal circadian conditions, showing better neuroprotection enabled by these gels in vivo (fig. S8).

In contrast to the TBS-treated mice, in which few axons regenerated (~200) to cross the lesion sites, the injection of either the LIF solutions or the LIF-laden gels led to significantly enhanced axon regeneration, particularly those ≥0.2 mm in length (Fig. 6). Moreover, the groups treated with the LIF-laden gels, under both normal circadian and dark conditions, displayed a great number of regenerated axons ≥0.2 mm in length (light, ~1600; dark, ~1800), more than those treated with the LIF solutions (~900), showing that the delivery of LIF by these protein gels is able to promote axon regeneration (Fig. 6). Although the influence of LIF-laden gels on STAT3 phosphorylation was almost indistinguishable under normal circadian (light) and dark conditions (Fig. 6), axon regeneration, especially when...
it comes to longer axons (0.5 mm), was affected by the light conditions. After the injection of the LIF gels, the dark-reared mice, but not those under normal circadian conditions, exhibited robust axon regeneration, in which the number of regenerated axons (0.5 mm) was three times that in the mice treated with the LIF solutions (Fig. 6). Note also that the administration of these protein gels led to negligible inflammation in the injured mice, highly indicative of their suitability for more in vivo applications. Together, these results demonstrated the capability of these metal-coordinated protein hydrogels for sustained delivery of neuroprotective proteins, showcasing their potential in neuroregeneration.

In summary, we have reported the creation of injectable, photoresponsive protein hydrogels using metal-directed assembly of His6-tagged CarHC. Because of their chemo/photoinduced phase transitions, these protein materials have allowed for facile encapsulation and controllable release of living cells and bioactive proteins. The gels decorated with neuroprotective cytokines have proven effective in enhancing neuronal survival and promoting axon regeneration in vivo, thus pointing to a new strategy for addressing the challenges facing regenerative neurobiology.

MATERIALS AND METHODS

Gene construction and protein expression

The *carHC* gene was purchased as a gBlocks gene fragment from Integrated DNA Technologies. pQE80l::His6-SpyTag-ELP-carHC-ELP-SpyTag (ACA) was constructed by replacing the middle RGD with carHC in pQE80l::AA (50). Sac I and Spe I restriction sites were used as described previously. *E. coli* strain DH5α was used for plasmid amplification.

*E. coli* strain BL21(DE3) was the bacterial host for protein expression. The bacterial cells were grown in LB at 37°C to the mid-log phase (optical density at 600 nm ~ 0.6 to 0.8), followed by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside (Sangon Biotech) to induce protein expression at 37°C. After 4 hours, cells were harvested and flash-frozen in liquid N2 before protein purification. The proteins were purified using HisTrap columns (GE Healthcare Inc.). The purified proteins were dialysed against Milli-Q water control. The light intensity was measured by Extech Light Meter LT300. To determine the amounts of GFP released into the supernatant, aliquots (10 μl) were taken at different time points, followed by superimposition of TBS (10 μl) each time to keep the supernatant volume constant, until the gels were fully dissolved in TBS. The GFP concentration was determined using a Variskan LUX multimode microplate reader (Thermo Fisher Scientific). Protein encapsulation and release with Zn2+-coordinated gels were achieved by following a procedure similar to that of the Co3+ gels, except that the Zn concentration was 4.8 mM and that the oxidation was skipped. All measurements were performed in triplicate. The released GFP concentration was determined using the equation below

$$\text{Percentage of GFP released} = \frac{90A_n + 10^2\text{Ext} \times 100}{90A_5 + 10^2\text{Ext} \times 100}$$

$A_n$ is the GFP fluorescence intensity of the sample taken at time point $n$ ($1 \leq n \leq 5$; $A_0 = 0$). Please note that the gels were fully dissolved in TBS at the end point ($n = 5$), which can be considered as complete protein release (100%). The total reaction volume (gel +...
of the Zn$^{2+}$-coordinated ACA with 1 ml trypsin solution (Sangon Biotech) followed by addition of passaged every 3 days. At 70 to 80% confluence, cells were detached.

**Encapsulation and light-induced release of 3T3 fibroblasts and hMSCs**

NIH 3T3 fibroblasts were cultured in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) (Sangon Biotech) supplemented with 10% (v/v) fetal bovine serum (Sangon Biotech), 1% (v/v) penicillin-streptomycin (Sangon Biotech), and 1% (v/v) nonessential amino acids (Sangon Biotech) in a 5% CO$_2$ atmosphere at 37°C and passaged every 3 days. At 70 to 80% confluence, cells were detached with 1 ml trypsin solution (Sangon Biotech) followed by addition of 2 ml DMEM to neutralize the trypsin. Around 60,000 cells were pelleted and resuspended with 27 µl of the Zn$^{2+}$-coordinated ACA solution in DMEM. The resulting cell suspension was then placed on a cell culture dish with a coverslip bottom. Gelation was initiated by adding 3 µl of AdoB$_{12}$ (25 mM in DMSO). The gel was cured in the dark for 45 min and then was immersed with the culture medium. After 12-hour incubation, the 3D culture was washed with TBS. The standard live/dead staining assays (Thermo Fisher Scientific) were performed on the encapsulated cells within the gel, followed by light-induced cell release and fluorescence imaging.

The cell release was induced by either blue light (480 nm) generated by the Lumen Dynamics XT640-W with a Nikon fluorescence isothiocyanate band-pass filter or white light (bright-field) emitted from a halogen lamp in the microscope. The power intensity of blue light was ~1.35 W/cm$^2$, determined by Thorlabs PM100D Compact Power and Energy Meter Console with S121C Standard Photodiode Power Sensor. The cell-laden gels were rinsed and then immersed using DMEM. On light exposure, the release of cells was recorded live with an epifluorescence microscope (Eclipse Ni-U; Nikon) or with an optical microscope equipped with a camera (Olympus CKX41). Cell viability was quantified using Fiji software, according to the protocol provided by BRTI Life Sciences. For each experiment, at least two identical gels from the same batch of proteins were prepared and tested for cell encapsulation under the same condition. At least three independent experiments were performed for each set of cell culture studies to ensure consistency.

**Animals and surgeries**

All animal experiments were performed in compliance with animal protocols that were approved by the Animal and Plant Care Facility at the Hong Kong University of Science and Technology. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/kg) for all surgical procedures. For the intravitreal injection, with a Hamilton microsyringe, LIF solutions (1.5 µl), LIF-laden gels (1.5 µl), control gels (LIF-free), or the vehicle (TBS) was injected into the vitreous bodies of the C57BL/6 mice; both LIF-free gels and LIF-free solution (TBS) were used as control. The injection was done in the darkroom with an infrared lamp. The mice were kept in the dark or in the normal circadian equipped with white LED light, of which the light intensity was determined to be ~0.3 klux by Extech Light Meter LT300.

To create the optic nerve crush, the optic nerve was gently excised from an incision on the conjunctiva and then was crushed behind the optic disk using fine forceps. After the optic nerve crush, the mice were given an appropriate dose of LIF-free or LIF-containing gel/solution via intravitreal injection as described above and were then kept alive for 2 weeks for axon regrowth. To visualize the axons of RGCs, 1.5 µl of cholera toxin subunit B, Alexa Fluor™ 488 conjugate (CTB-488) (2 µg/µl; Invitrogen) was injected into the vitreous body to label the regenerating axons 2 days before the mice were sacrificed.

**Immunostaining of retinas and optic nerves**

Mice were given a lethal dose of anesthesia (ketamine and xylazine) and then perfused with phosphate-buffered saline (PBS) and 4% paraformaldehyde (PFA) (Sigma-Aldrich). Retinas and optic nerves were dissected, fixed in 4% PFA overnight, and then dehydrated in 30% sucrose for 6 hours. The resulting retinas and optic nerves were embedded into optimal cutting temperature compound (Sakura) at −80°C and then cryosectioned at ~20°C (25 µm for retinas and 8 µm for optic nerves). These sections were blocked with 0.1% Triton X-100 in 4% normal goat serum for 30 min and then incubated overnight with the primary antibodies, including Tuj1 (neuron-specific class III beta-tubulin) (BioLegend, catalog no. 801202; mouse monoclonal antibody), p-STAT3 705 (Cell Signaling Technologies, catalog no. 9145; rabbit monoclonal antibody), and Alexa Fluor 488 (Invitrogen, catalog no. A-11094; rabbit polyclonal antibody). The sections were washed with PBS (three times) and then incubated with suitable secondary antibodies (Invitrogen) for 2 hours. After wash with PBS (three times), the sections were mounted on coverslips for imaging.

**Quantification of axon regeneration**

To quantify the number of regenerated axons, the optic nerve was dissected and placed longitudinally for cryosection. The serially collected optic nerves were stained with the Alexa Fluor 488 antibody and imaged under a confocal microscope. The formula, $\Sigma a = \pi r^2 \times \left[\text{average axon numbers/mm} / t\right]$, was used to count regrown axons at a defined distance (0.2 or 0.5 mm) to the lesion site, where $r$ is the radius of the optic nerve at the counting site, average axon numbers per millimeter represents the average numbers of axons per millimeter (in width) of nerve, and $t$ is the section thickness (8 µm).

**Quantification of RGCs**

The percentage of p-STAT3 705–positive RGCs was determined by co-staining the retinas harvested from intact mice with the Tuj1 and p-STAT3 705 antibodies. The survival of RGCs in optic nerve–crushed mice was determined by Tuj1 staining. After staining, the sections were mounted on coverslips for confocal imaging. A dozen images from each retina, covering both the peripheral and central regions, were captured for quantification.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/41/eabc4824/DC1

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