Drug Delivery

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Magnetically Guided Protein Transduction by Hybrid Nanogel Chaperones with Iron Oxide Nanoparticles

Riku Kawasaki, Yoshihiro Sasaki, Kiyofumi Katagiri, Sada-atsu Mukai, Shin-ichi Sawada, and Kazunari Akiyoshi**

Abstract: *Protein pharmaceuticals show great therapeutic promise, but effective intracellular delivery remains challenging. To address the need for efficient protein transduction systems, we used a magnetic nanogel chaperone (MC): a hybrid of a polysaccharide nanogel, a protein carrier with molecular chaperone-like properties, and iron oxide nanoparticles, enabling magnetically guided delivery. The MC complexed with model proteins, such as BSA and insulin, and was not cytotoxic. Cargo proteins were delivered to the target HeLa cell cytosol using a magnetic field to promote movement of the protein complex toward the cells. Delivery was confirmed by fluorescence microscopy and flow cytometry. Delivered β-galactosidase, inactive within the MC complex, became enzymatically active within cells to convert a prodrug. Thus, cargo proteins were released from MC complexes through exchange interactions with cytosolic proteins. The MC is a promising tool for realizing the therapeutic potential of proteins.*

Protein pharmaceuticals are promising for combating human diseases including cancer, metabolic disorders and autoimmune diseases.[1] More than 130 proteins or peptides have been approved for clinical use.[2] Compared with conventional small molecule drugs, protein pharmaceuticals have several advantages, derived from their specific and multiple functions and high biocompatibility.[1] Furthermore, therapy with proteins can avoid issues encountered with other types of agents, such as permanent or random genetic alterations of cells caused by nucleic acid drugs. Thus, protein pharmaceuticals, which have only temporary therapeutic effects, are often safer and more efficient alternatives than gene therapies.[1]

Despite their fascinating potential, protein pharmaceutical development remains challenging because of their physical and chemical instabilities, necessitating high doses. To date, many nanocarriers for protein pharmaceuticals have been designed and synthesized, including lipids,[3] polymers,[4] nanogels,[5] and inorganic nanoparticles.[6] In addition, most protein pharmaceuticals (for example, antigens, cytokines, and transcription factors) exert their functions by binding to the cell membrane surface or to extracellular targets.[1] However, recent progress in molecular biology has indicated that intracellular delivery of proteins could increase their potential uses in cancer therapy or regenerative medicine.[2] Accordingly, effective systems to deliver proteins into the cell, that is, protein transduction systems,[17] are needed to fully realize the therapeutic potential of proteins.

Recently, many nanocarriers with magnetic properties have been developed to achieve efficient intracellular drug delivery. For instance, magnetic nanoparticles coated with lipid,[8] polymers,[9] and proteins[10] were designed as magnetically guided delivery systems. In particular, transfection methods using magnetic nanoparticles, known as magnetofection,[11] have been extensively investigated and their efficiencies confirmed in various cell lines. However, few examples have been described using covalent attachment of proteins to magnetic nanoparticles in magnetically guided protein delivery systems[12] because maintaining proteins in their innate and functional form would be difficult to achieve with such approaches.[13]

To address these issues in protein transduction, two approaches were employed here. One was to develop techniques to handle proteins, inspired by molecular chaperones. The chaperone-like function is defined as one that traps proteins without causing their aggregation and releases them in native form. This is an important concept for the formulation of a protein pharmaceutical. The other approach employed here was efficient intracellular delivery using an external magnetic field, which is generally harmless to both recipient cells and cargo proteins. The basic idea for magnetically guided protein transduction is shown in Figure 1. Specifically, magnetically guided nanocarriers were designed and synthesized by hybridizing an amphiphilic polysaccharide nanogel with magnetic nanoparticles. The hybrid (magnetic nanogel chaperone, MC) was anticipated to have the functions of a protein carrier nanogel with magnetic properties.

We have developed physically crosslinked nanogels as protein nanocarriers.[14] For example, hydrophobic polysaccharides, such as cholesterol-bearing pullulan, form self-assembled nanogels in water through physical crosslinking by hydrophobic cholesterol groups.[15] One of the most attractive characteristics of nanogels is their molecular chaperone-like function, enabling capture of proteins within their polymer matrix through hydrophobic interactions. In addition, the dynamic properties of self-assembled nanogels enable release

[*] R. Kawasaki, Dr. Y. Sasaki, Dr. S. Mukai, Dr. S. Sawada, Prof. Dr. K. Akiyoshi
Department of Polymer Chemistry, Graduate School of Engineering
Kyoto University, Katsura, Nishikyo-ku, Kyoto (Japan)
E-mail: akiyoshi@bio.polym.kyoto-u.ac.jp
Dr. K. Katagiri
Graduate School of Engineering, Hiroshima University
1-4-1 Kagamiyama, Higashi-Hiroshima (Japan)
Dr. S. Mukai, Dr. S. Sawada, Prof. Dr. K. Akiyoshi
JST-ERATO, Akiyoshi Bio-nanotransporter Project
Kyoto University Katsura, Nishikyo-ku, Kyoto (Japan)

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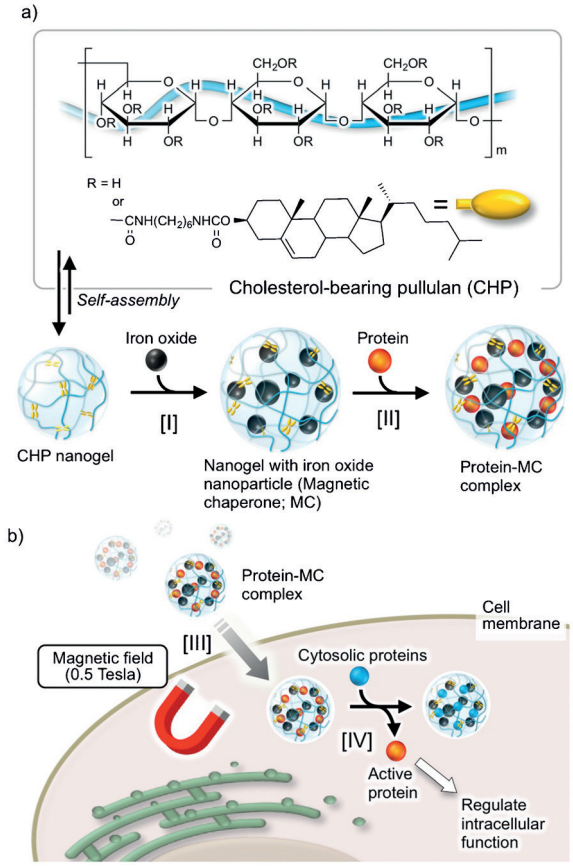

Figure 1. Design of a magnetically guided protein transduction system. a) Preparation of protein/MC complexes. [I] The MC was prepared by mixing a nanogel with hydrophobized iron oxide nanoparticles. [II] Proteins were encapsulated within the polymer matrix of MC. b) Delivery of proteins by MC to the cytosol. [III] Movement of protein/MC complexes toward cells, guided by an external magnetic field. [IV] Release of proteins within the cell by exchange reactions of cargo proteins with cytosolic proteins.

of the proteins from the nanogel through exchange reactions with other proteins in the bulk phase.[14a] The "catch and release" chaperone function allowed nanogels to act as protein nanocarriers in the development of a cancer vaccine (in Phase 1 and 2 clinical trials), a nasal vaccine,[16] and delivery systems for cytokines such as interleukin-1[17] and bone morphogenetic protein.[18] We previously reported facile preparation of hybrids of nanogels and iron oxide nanoparticles with excellent theranostic properties, including T_2 relaxivity, for use as a magnetic resonance imaging (MRI) contrast agent, and a heat generator for magnetic hyperthermia.[19] These preliminary results encouraged us to develop a nanocarrier to deliver protein pharmaceuticals into cells using an external magnetic field, which can induce movement of the nanocarrier toward targeted cells to deliver the cargo proteins.

Preparation and detailed characterization of the hybrid whas been reported elsewhere.[18] Briefly, the hybrid was prepared by mixing an aqueous nanogel suspension formed with cholesterol-bearing pullulan (CHP; 100 μg mL^{-1}) and an aliquot of hydrophobized iron oxide nanoparticles suspended in tetrahydrofuran[19] (the hydrodynamic diameter of the magnetic nanoparticles was 12 nm and their polydispersity index was 0.012).

At first, to examine the appropriateness of the MC as an intracellular delivery carrier, the viability of HeLa cells in the presence of the MC was evaluated using a modified MTT assay. Figure 2 a shows the viability of cells treated with a pure CHP nanogel (100 μg mL^{-1}) or a CHP nanogel (100 μg mL^{-1}) mixed with 1, 10, or 100 μg mL^{-1} iron oxide nanoparticles (MC1, 10, and 100, respectively; Supporting Information,

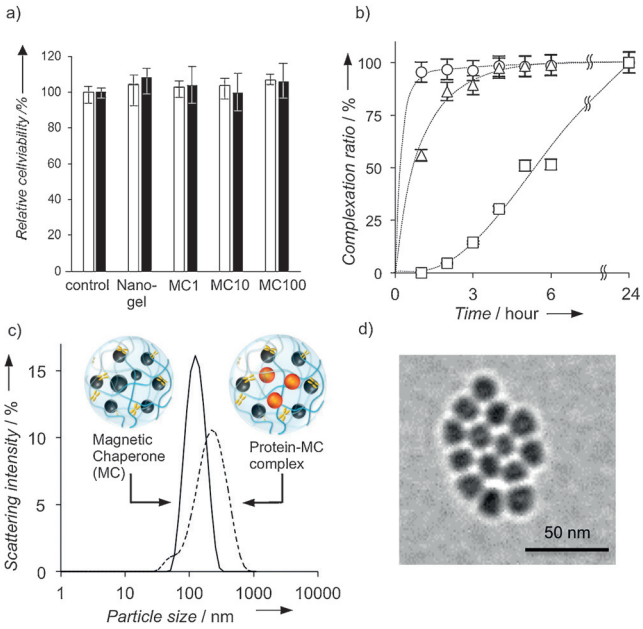

Figure 2. Characterization of MC and its interactions with proteins. a) Cytotoxicity of MC in the absence (white) or presence (black) of a magnetic field. HeLa cells were treated with CHP nanogel or MC. The concentration of CHP nanogel was fixed at 100 μg mL^{-1}. Three independent experiments were performed in triplicate. b) Complexation between MC (100 μg mL^{-1}): MC1 (○), MC10 (△), or MC100(□), and Alexa488-labeled BSA (17 μg mL^{-1}), which were estimated by fluorescence spectroscopy after magnetic separation. Three independent experiments were performed in triplicate c) Size distribution of MC1 and MC1/BSA complexes. MC was dispersed in PBS (25 °C, pH 7.4). d) TEM images of MC1/BSA complex (MC1, 100 μg mL^{-1}; BSA: 17 μg mL^{-1}) in PBS. Scale bar = 50 nm.

Figure S1 and Table S1). No apparent cytotoxicity was observed even at the highest concentration of iron oxide nanoparticles after irradiation of magnetic field for 24 h. This suggests that the MC could be used as non-toxic nanocarrier, even under magnetic field irradiation.

Protein complexation with the MC was evaluated using fluorescently labeled bovine serum albumin (BSA, 68 kDa) as a model protein. BSA (17 μg mL^{-1}) was incubated with MC (100 μg mL^{-1}) for 24 h. Proteins incorporated with the MC were separated from free proteins in a magnetic field and the fluorescence intensity of the supernatant was measured to quantify the complexation ratio (%), expressed as the amount of incorporated protein relative to the total protein (Fig-

ure 2b). When BSA was mixed with MC1, all of the protein was incorporated into the nanogel within 3 h. In addition, the complexation rates depended on the ratio of nanogel to iron oxide nanoparticles, indicating that the protein binding capacity of the MC can be tuned by changing the incorporated concentration of iron oxide nanoparticles.

Dynamic light scattering analysis showed that the size of the MC1 increased from 100 to 180 nm after complexation with BSA (Figure 2c). However, the zeta-potential did not significantly change (from −1.0 to −0.4 mV), although MC interacted with anionic BSA under the pH conditions applied. These results indicate that BSA was encapsulated within the nanogel, shielding its anionic charge. Figure 2d shows representative morphological images of a MC/BSA complex observed by transmission electron microscopy, indicating that MCs maintained their spherical morphology after complexation with proteins.[17]

The performance of the MC as a magnetically guided intracellular protein nanocarrier was investigated by confocal laser scanning microscopy (CLSM). HeLa cells were exposed to a MC/BSA complex (100 μg mL^{-1}; BSA: 17 μg mL^{-1}) for 24 h and magnetic fields (ca. 0.5 T) were applied towards the cells from the bottom of the culture dish. The subcellular distributions of delivered carriers and fluorescently labeled cargo proteins were observed by CLSM. Fluorescence from MC (red) and BSA (green) was detected in the cytosol when magnetically induced protein delivery was performed with MC1 (Figures 3a and S2c). In contrast, fluorescence was not detected in cells incubated with conventional nanogels, or with MC1 in the absence of a magnetic field (Figure S1). Flow cytometry also showed that proteins were effectively delivered by MC under a magnetic field (Figure 3b).

The intracellular uptake efficiency of MC under the influence of a magnetic field depended on the strength and direction of the magnetic field toward the cells (Figure S3). The magnetically induced protein delivery systems with MC yielded, on average, a 100-fold enhancement of MC and BSA uptake compared with the same carrier in the absence of a magnetic field. The fluorescence from MC1 and cargo proteins dispersed separately in the cytosol after 24 h. In addition, the cellular uptake was not inhibited by incubation at 4 °C (Figure S4), suggesting that MCs were taken up by following two energy-independent pathways. One pathway is the direct crossing of the plasma membrane by spearing the lipid bilayer, and the other is endocytosis-like uptake resulting from magnetic energy. To further examine the release of proteins from MCs, the co-localization of MC and BSA was estimated. As expected, the co-localization ratio was decreased from 80% at 4 h to 30% at 24 h. These results indicate that the proteins were released from MC within cells after 24 h, because of an exchange reaction between cargo and cytosolic proteins.

To demonstrate protein release from MC, the MC/BSA complex (MC1, 100 μg mL^{-1}; BSA, 17 μg mL^{-1}) was co-incubated with highly concentrated serum proteins. Release of incorporated proteins was quantified after magnetic separation of complexed protein to free protein. As shown in Figure 3c, BSA was not released from MC in PBS, whereas BSA was gradually released from MC in the presence of

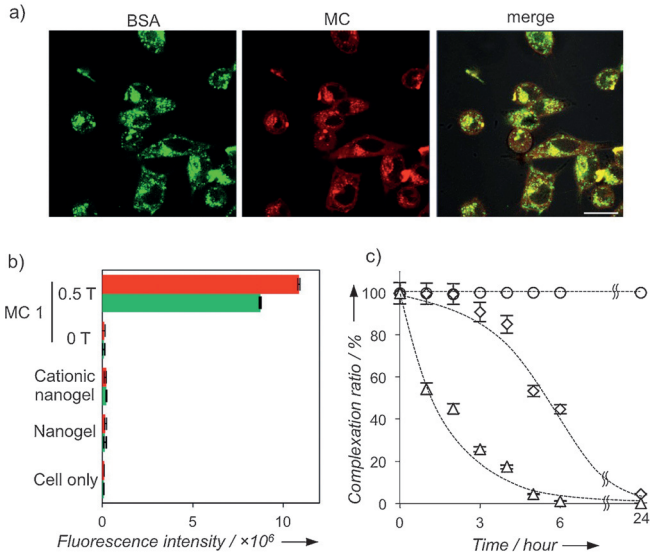

Figure 3. Cell uptake of MC/Alexa488-BSA complexes under irradiation in a magnetic field. a) Confocal laser scanning microscopic images of HeLa cells after incubation for 24 h with a complex of rhodamine B-labeled MC1 with Alexa488-labeled BSA under a magnetic field (MC1, 100 μg mL^{-1}; BSA, 17 μg mL^{-1}). The scale bar indicates 20 μm. b) Mean fluorescence of rhodamine B-labeled MC1 (red) or Alexa488-labeled BSA (green) accumulated in HeLa cells, as determined by flow cytometry after 24 h incubation with nanogel/BSA complexes, or MC1/BSA complexes. c) Protein release in FBS, mimicking the intracellular environment. After complexation with BSA, PBS (○) or FBS (10% ◇, 20% △) were added to the complex and labeled protein release was determined by fluorescence spectroscopy after magnetic separation. Three independent experiments were performed in triplicate.

concentrated serum proteins in fetal bovine serum (FBS). The protein release efficiency was accelerated by increasing the concentration of serum proteins with FBS (10% FBS to 20% FBS). The serum proteins were detected in MC after mixing with FBS and incubation for 24 h by polyacrylamide gel electrophoresis (SDS-PAGE, Figure S5). These results indicate that the cargo proteins were replaced with serum proteins through an exchange reaction. Generally, the cytosol is filled with highly concentrated proteins. Accordingly, MC released cargo proteins within the cytosol through a protein exchange reaction. Similar protein release was observed with insulin as a lower molecular weight model protein (Figure S6).

The function of proteins delivered by MC to the cytosol was investigated with a hydrolytic enzyme, β-galactosidase (β-gal). When a solution of β-gal (0.42 μg mL^{-1}) and MC (100 μg mL^{-1}) was incubated for 24 h, 52% of β-gal was complexed with MC. The MC/β-gal complex was then separated from free β-gal and its enzymatic activity evaluated by monitoring the hydrolysis of the galactosyl moiety of non-fluorescent Tokyo-green β-gal (10 nM) to fluorescent Tokyo-green. As shown in Figure 4a, β-gal was inactivated by complexation with MC. After addition of FBS to the complex, however, β-gal recovered its enzymatic activity, probably because of its release and refolding following protein exchange reactions with serum proteins in FBS. These results showed that MC acted as an artificial molecular chaperone,

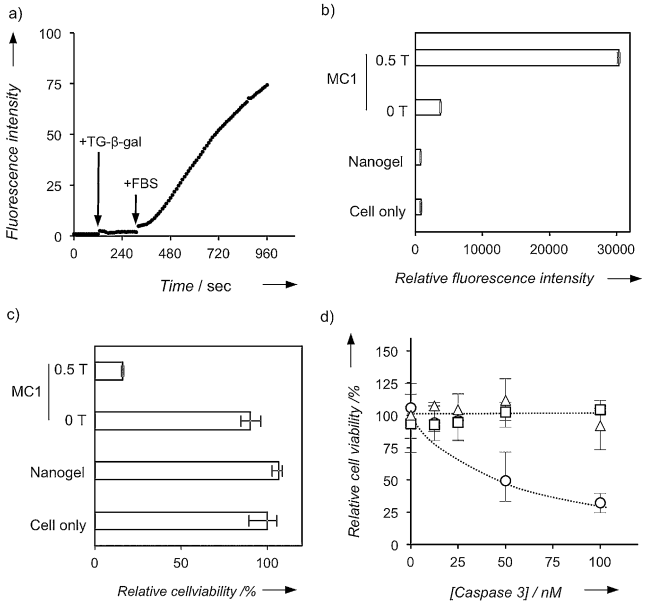

Figure 4. Chaperone-like activity of MC through protein exchange reactions and protein transduction using MC. a) Recovery of enzymatic activity of β-gal after its release from MC. MC/β-gal complex was mixed with Tokyo green β-gal (10 nM) and 20% FBS. Each reagent was added at 60 or 300 s. b) Protein transduction efficiencies, determined by measuring fluorescence intensity of Tokyo-green β-gal. HeLa cells were incubated with nanogel/β-gal complex, MC1/β-gal complex, or MC1/β-gal complex under magnetic field. The concentration of β-gal was fixed at 0.42 μg mL^{-1}. Tokyo-green β-gal (10 μM) was added after 24 h incubation. c) Activation of the anticancer prodrug 5-FUR-β-gal by MC/β-gal complexes, as determined by HeLa cell viability. Relative cell viabilities were determined after treatment 24 h with nanogel/β-gal complexes, MC1/β-gal complexes in the presence of magnetic field or absence of an external magnetic field. Three independent experiments performed in triplicate. The concentration of nanogel and β-gal were fixed at 10 μg mL^{-1} and 0.42 μg mL^{-1}, respectively. The standard deviations of three independent measurements are shown. Three independent experiments performed in duplicate. d) Delivery of caspase-3 into CT26 cells. CT26 (murine colon cancer cells) were treated with free caspase-3 (□), MC/caspase-3 without irradiation of magnetic field (△), and with irradiation of magnetic field (○). After 24 h, cell viabilities were evaluated by WST-8 assay. The concentration of MC was fixed at 100 μg mL^{-1}. Three independent experiments performed in triplicate.

enabling it to catch and release cargo proteins through exchange reactions with cytosolic proteins.

Protein transduction efficiencies were directly evaluated by monitoring enzymatic activity of MC-delivered β-gal within Hela cells. The complex of β-gal with MC was incubated with Hela cells for 24 h under a magnetic field and then CLSM observations were carried out. No fluorescence was detected in the case of free β-gal, the nanogel/β-gal complex, or the MC/β-gal complex without a magnetic field (Figure S7). However, when β-gal was delivered to HeLa cells using MC under a magnetic field, the fluorescence from Tokyo-green, which was generated by the hydrolysis of Tokyo-green β-gal, was clearly observed throughout the cytosol (Figure S7d or f). In contrast, lower fluorescence emission from Tokyo-green was observed when β-gal was delivered from a cationic nanogel as a positive control.

The enzymatic activity was also quantified by flow cytometry. The fluorescence intensity from cells treated with a MC1/β-gal complex under a magnetic field was 100 times higher than without a magnetic field (Figure 4b). The enzymatic activity within cells gradually decreased over 72 h to 8.5% (Figure S8). The disappearance of protein activity within cells is advantageous for protein therapies that require temporal pharmaceutical effects.

A prodrug assay was conducted using β-gal conjugated with cytotoxic 5-fluorouridine (5-fluorouridine-5′-*O*-β-galactopyranoside, 5-FUR-β-gal) as a model prodrug substrate. When the galactosyl moiety of 5-FUR-β-gal was hydrolyzed by β-gal, the product 5-FUR showed dose-dependent cytotoxicity, whereas 5-FUR-β-gal was not cytotoxic (Figure S9). After magnetically guided protein transduction using MC, an apparent cytotoxicity from 5-FUR for Hela cells was observed (Figure 4c). Thus, the delivered β-gal showed enzymatic activity within cells to convert non-cytotoxic 5-FUR-β-gal into cytotoxic 5-FUR.

Finally, the utility of MC for DDS was assessed by delivering caspase-3, which is a known cytosolic protease that participates in a signaling pathway leading to programed cellular death (apoptosis). Caspase-3 or the MC/caspase-3 complex was incubated with murine colon cancer cells (CT26 cells) for 24 h. An apparent cytotoxicity was observed only when caspase 3 was delivered by MCs under the influence of a magnetic field (Figure 4d). In addition, flow cytometry showed that cell destruction was induced through an apoptosis pathway by the delivered caspase-3 (Figure S10).

In conclusion, we demonstrated a facile magnetically guided protein transduction method using polysaccharide nanogels with iron oxide nanoparticles. The chaperone-like function of nanogels and the magnetic properties of iron oxide enabled highly efficient delivery of proteins in their active forms to target cells. Consequently, our strategy has great potential as a key technique for protein transduction. We believe that supramolecular chemistry-based approaches such as ours have unique features applicable to delivering a wide range of proteins for protein-based therapies or regenerative medicine in the near future.

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Keywords: crosslinked nanogels · drug delivery · organic–inorganic nanomaterials · protein-based therapies · protein transduction

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