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# Cell-penetrating macromolecules: Direct penetration of amphipathic phospholipid polymers across plasma membrane of living cells

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ABSTRACT

Nanoscaled materials are normally engulfed in endosomes by energy-dependent endocytosis and fail to access the cytosolic cell machinery. Although some biomolecules may penetrate non-endocytically or fuse with plasma membranes without overt membrane disruption, to date no synthetic macromolecule of comparable size has been shown to exhibit this property. Here, we discovered mechanism of direct cell membrane penetration using synthetic phospholipid polymers. These water-soluble amphiphilic phospholipid polymers enter the cytoplasm of living mammalian cells *in vitro* within a few minutes without overt bilayer disruption even under conditions where energy-dependent endocytic uptakes are blocked. Furthermore, targeted cytosolic distribution to cell organelles was achieved by selecting specific fluorescent tags to the polymers. Thus, the phospholipid polymers can provide a new way of thinking about access to the cellular interior, namely direct membrane penetration.



## 1. Introduction

Nanomaterials are of great interest for their potential biomedical applications as imaging tools [1,2] therapeutic agents [3], and drug/gene delivery carriers [4,5]. However, the plasma membrane of eukaryotic cells constitutes a highly selective permeability barrier that prevents the passage of nanomaterials, allowing only small, uncharged molecules to gain access to the cell interior. Thus, translocation of nanomaterials across plasma membranes is currently one of the most actively researched topics in nanobiology. The most common mechanism of cellular uptake for nanomaterials is endocytosis, an energy-dependent process in which the nanomaterial is trapped within endosomes that carry ingested material into the cellular interior while the engulfed materials do not gain access to the cytosol [6]. Cationically charged nanomaterials, such as dendrimers [7], quantum dots [8], and polymeric micelles [9], can pass through anionic plasma membranes by causing transient pore formation, a process that is also associated with cytotoxicity. Alternatively, nanoparticles coupled with exogenous cell-penetrating peptides (CPPs) have been designed to explicitly disrupt the endolysosomal membrane, thereby facilitating entry into the cytosol [10,11]. CPP-conjugated nanomaterials may be capable of directly penetrating the membrane without overt lipid bilayer disruption. To the best of our knowledge, however, no synthetic macromolecules larger than a few nanometers in size can penetrate directly through the plasma membrane without disturbing the integrity of the biological barriers.

Phospholipid molecules play a crucial role in determining the composition of the cytosol by forming a bilayer structure with barrier properties and mass transport function. In addition, they form a bio-inert surface in the cell membrane that facilitates highly efficient, selective, and specific biological reactions. By mimicking the chemical functionality as well as the molecular structure of phospholipids in the plasma membrane, we have synthesized phospholipid polymers comprising 2-methacryloyloxyethyl phosphorylcholine (MPC) as a polar group and *n*-butyl methacrylate (BMA) as a hydrophobic part [12]. The poly(MPC-co-BMA) (PMB), which forms nanoassemblies (polymeric aggregates) or nanoparticles in aqueous media depending on the degree of hydrophobicity and molecular weight of the polymers, has considerable potential for therapeutic nanomaterials [13]. Previously, a PMB carrying an antineoplastic drug was demonstrated to exhibit antitumor effects on cancer cells without cytotoxicity both *in vitro* and *in vivo* [14–16]; however, the mechanism whereby the drug in the PMB was delivered across the plasma membrane of cells remains

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poorly understood. In this study, we discovered direct penetration mechanism across the plasma membrane of living mammalian cells using fluorescence-tagged PMBs.

## 2. Materials and methods

### 2.1. Materials

MPC, which was synthesized as previously described [17], was purchased from NOF (Tokyo, Japan). The commercially available reagents *n*-butyl methacrylate (BMA; Nacalai Tesque, Tokyo, Japan), methacryloxyethyl thiocarbonyl rhodamine B (MTR; Polysciences, PA, USA), *o*-methacryloyl Hoechst 33258 (Polysciences), fluorescein *o*-methacrylate (Polysciences), 1-pyrenylmethyl methacrylate (Polysciences), t-butyl peroxyneodecanoate (Perbutyl-ND; NOF), and 2,2′-azobisisobutyronitrile (AIBN; Kanto Chemical Co., Tokyo, Japan) were of extra pure grade and used without further purification. Unless stated otherwise, all the other materials were purchased from commercial sources and used as received.

### 2.2. Synthesis of fluorescent polymers

The poly(MPC-*co*-BMA) with covalent labeling of rhodamine B (rhoPMB) was synthesized using a free radical polymerization technique with Perbutyl-ND as an initiator. For rhoPMB30 synthesis, MPC (2.66 g, 9.00 mmol), BMA (2.99 g, 21.0 mmol), and MTR (20.5 mg, 30.0 μmol) were dissolved in ethanol (30.0 mL) and argon gas was bubbled through the resulting solution for 30 min in order to remove dissolved oxygen. Perbutyl-ND (70 wt% in hydrocarbons, 0.367 g, 1.05 mmol) was then added to the solution and the temperature was elevated to 60 °C for 12 h to induce polymerization. Thereafter, the polymer was reprecipitated twice in diethylether/chloroform (7/3 v/v). The polymer was then dialyzed against water through a regenerative cellulose membrane (MWCO, 3.5 kDa) for 7 days, and subsequently ultrafiltrated in methanol/water (7/3 v/v) for 7 days (MWCO, 5.0 kDa) to remove unreacted monomers and absorbed dyes. rhoPMB50, rhoPMB80, and rhodamine B-labeled poly(MPC) (rhoPMPC; MPC/MTR = 100/0.1 mol/mol) were synthesized using the same method. Hoechst 33258-tagged PMB30 (hoechstPMB30) and FITC-tagged PMB30 (fitcPMB30) were synthesized using the same method used to synthesize rhoPMB30, but using *o*-methacryloyl Hoechst 33258 and fluorescein *o*-methacrylate, respectively, instead of MTR. High molecular weight rhoPMB polymers were synthesized using a free radical polymerization technique with AIBN (4.90 mg, 30.0 μmol) instead of perbutyl-ND as an initiator, and were purified using the same method described above. The high molecular weight polymers were dialyzed against water through a regenerative cellulose membrane (MWCO, 50 kDa).

### 2.3. Cell culture

The human Caucasian hepatocyte carcinoma (HepG2) cell line (RIKEN BioResource Center, Ibaraki, Japan) was seeded on tissue culture polystyrene ($1.0 \times 10^4$ cells/cm$^2$) in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco), and penicillin/streptomycin (100 μg/mL, Gibco) at 37 °C in 5% $CO_2$. Sub-confluent cultures (70–80%) were passaged using 0.25% trypsin/EDTA at 37 °C in 5% $CO_2$. HeLa cells (RIKEN BioResource Center) were passaged at sub-confluency in DMEM containing 10% FBS.

### 2.4. Cellular uptake of fluorescent-labeled polymers

Cells were seeded into glass-bottom dishes (0.12–0.17 mm thick; Matsunami, Osaka, Japan) at $5.0–6.0 \times 10^4$ cells/mL in 1.0 mL of culture medium and grown for 1 day before incubation with the fluorescent polymers. The cells were then rinsed 3 times with DPBS. The medium was subsequently exchanged with fresh warm serum-free or serum-containing (10% FBS) DMEM and fluorescent polymers were added from a concentrated stock solution in DMEM at a final concentration of 1.0 mg mL$^{-1}$. Cells were incubated with fluorescence-tagged polymer at 37 °C in 5% $CO_2$ for the desired length of time. After incubation with the polymer, the cells were rinsed 3 times with DPBS and placed in 1.0 mL of DMEM for live cell imaging. Staining of cells under conditions of energy depletion was performed by incubating the cells with 3.0 mM sodium azide and 50 mM 2-deoxy-D-glucose in DPBS (ATP depletion solution). Cells were pre-incubated in ATP depletion solution at 37 °C for 30 min and then incubated with 1.0 mg mL$^{-1}$ fluorescent polymers in ATP depletion medium at 37 °C in 5% $CO_2$. The cells were then washed 3 times with DPBS and placed in 1.0 mL of DMEM. Staining under conditions inhibitory to the mechanism of active uptake by cells was performed by incubating at 4 °C. Cultured cells were washed 3 times with DPBS and precooled in serum-free DMEM at 4 °C for 30 min. The cells were then incubated in serum-free DMEM with 1.0 mg mL$^{-1}$ fluorescence-tagged polymer at 4 °C. Thereafter, the cells were rinsed 3 times with DPBS and placed in 1.0 mL of serum-free DMEM.

### 2.5. Confocal fluorescent microscopy studies

The polymers in living cells were imaged using an LSM-510 Meta confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with a 63× oil-immersion objective lens with a numerical aperture of 1.4 with respect to the beam waist. Confocal-DIC images were obtained by excitation with a 633-nm HeNe laser line. Fluorescent probes in Hoechst 33258-, FITC-, and rhodamine B-tagged polymers in the cells were excited with a 405-nm line (selected by an extra 405 ± 10 nm interference-based laser cleanup filter) of a blue diode laser, a 488-nm line of an Ar laser, and a 543-nm line of a HeNe laser, respectively. The excitation light was directed to the sample by a dichroic mirror (DC; HFT 405, 488, and 543, respectively). Each fluorescent light was sent through the DC and a long-pass LP 420 nm, band-pass BP 520–550 nm, and LP 560 nm emission filter to an analog photomultiplier tube used for detection. The detector gain was set to 650–850 units in the Zeiss software. Dark signal contributions to the images were effectively zeroed out by setting the proper detection offset value as provided in the Zeiss software. The image size was typically set to 512 × 512 or 1024 × 1024 pixels. Multi-stained cells were imaged using multi-track mode for the prevention of cross-talk. The full cellular thickness (approximately 11 μm) was scanned with the confocal microscope in 1.4-μm steps. Confocal Z-section scanning for imaging of the nucleus was conducted in 0.4-μm steps. The internalization of rhodamine B-tagged polymers into living cells was monitored by time-lapse imaging every 30 s up to 30 min at 37 °C. After rinsing 3 times with DPBS, cultured cells were placed in fresh warm serum-free DMEM and the glass-bottom dish was placed on a temperature control module under a 63× oil lens. Time-lapse imaging was started just after the fluorescent polymer was added from a concentrated stock solution in DMEM at a final concentration of 1.0 mg mL$^{-1}$.

### 2.6. Fluorescence correlation spectroscopy (FCS) studies

Scanning measurements were performed using an LSM 510 Meta microscope equipped with a ConfoCor 3 unit for FCS (Carl Zeiss). MTR and rhodamine B-tagged polymer were excited at 543 nm by the built-in HeNe laser, focused onto the sample by a 40× water-immersion objective lens (C-Apochromat) with a numerical aperture of 1.2 relative to the beam waist with $1/e^2$ radius $\omega_{xy} = 0.2$ μm. The pinhole in front of the avalanche photodiode was set to 78 μm. Fluorescence was detected by an avalanche photodiode in the ConfoCor unit. To select the detected spectral range, an LP 560-nm filter was used for the measurements. The fluorescence temporal signal was recorded, and the autocorrelation function $G(t)$ was calculated using the internal correlator. Curve fittings were carried out using ConfoCor, with a triplet state identified in the fitting, and with two-dimensional fitting. The waist radius ($\omega_1$) of the 543-nm laser lines was set at 0.20 μm on the basis of the known diffusion coefficient of rhodamine B in solution ($D = 28 \times 10^{-9}$ m$^2$/s). The effective diffusion coefficients of the fluorescent-tagged polymer ($D$) were determined using the calibrated beam waist and the diffusion time obtained from the autocorrelation curve via the equation $D = \omega_1^2/4\tau_D$, where $\tau_D$ is the diffusion time. The temperature dependence of the diffusion coefficient can be written in the form of the Stokes–Einstein equation, $R_H = k_BT/6\pi\eta D$, where $R_H$ denotes the hydrodynamic radius, $k_B$ Boltzmann's constant, $T$ the absolute temperature, and $\eta$ the solution viscosity. The autocorrelation curve was generated from 30 × 15-s data collections.

### 2.7. Subcellular distribution of the fluorescent polymers

HepG2 cells were pre-incubated with 100 nM of MitoTracker Green FM (Molecular Probes), which is a specific fluorescence dye for mitochondria, in DMEM containing 10% FBS for 24 h at 37 °C in 5% $CO_2$. After rinsing 3 times with DPBS, the stained cells were incubated in serum-free DMEM containing rhoPMB (1.0 mg mL$^{-1}$) at 37 °C in 5% $CO_2$ for 30 min. The cells were then rinsed 3 times with DPBS and placed in 1.0 mL of DMEM for imaging using an LSM 510 Meta confocal microscope operating in multi-track mode. MitoTracker was excited with a 488-nm line and imaged through band-pass BP 520–550-nm emission filter to the PMT.

### 2.8. Flow cytometry studies

HepG2 cells ($5.0 \times 10^5$) were seeded in 6-well culture plates and grown overnight in DMEM containing 10% FBS at 37 °C in 5% $CO_2$. After rinsing 3 times with DPBS, the cells were incubated with fluorescence-tagged polymers under the desired conditions. Thereafter, the cells were rinsed 3 times with DPBS and treated with trypsin/EDTA. The cells were then suspended in DMEM containing 10% FBS and analyzed immediately using an EPICS XL flow cytometer (Beckman Coulter, CA, USA) operating at 488-nm excitation and with either FL-2 or FL-3 emission filters. For the measurement of release of the fluorescence-tagged polymer from the cells, the suspension was incubated for 0–180 min at room temperature before flow cytometric analysis. A minimum of $1.0 \times 10^4$ cells were analyzed from each sample with florescence intensity.

### 2.9. Calcein-AM staining

Calcein-AM (10 μM in DPBS, Invitrogen) was pre-incubated with cells for 30 min in serum-containing DMEM (final concentration: 100 nM). Cells were then rinsed 3