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Endocytosis and Intracellular Trafficking Properties of Transferrin-Conjugated Block Copolypeptide Vesicles

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Abstract

Block polypeptides are an emerging class of materials that have the potential to be used in many biomedical applications, including the field of drug delivery. We have previously developed a negatively charged block copolypeptide, poly(L-glutamate)₆₀-*b*-poly(L-leucine)₂₀ (E₆₀L₂₀), which forms spherical vesicles in aqueous solution. Since these vesicles are negatively charged, they are minimally toxic toward cells. However, the negative charge also inhibits these vesicles from effectively being internalized by cells, which can be problematic as many therapeutics have intracellular targets. To overcome this limitation of the E₆₀L₂₀ vesicles, transferrin (Tf) was conjugated onto the vesicle surface, since the receptor for Tf is overexpressed on cancer cells. The enhanced uptake of the Tf-conjugated vesicle was verified through confocal microscopy. Furthermore, endocytosis and immunostaining experiments confirmed that the Tf conjugated on the vesicle surface plays a critical role in the internalization and subsequent intracellular trafficking behavior of the vesicles.

Keywords

vesicles; block copolypeptides; transferrin; immunostaining; clathrin-mediated endocytosis

Introduction

Block copolypeptide vesicles are a new class of polymeric vesicles that have the properties to become effective carriers for therapeutics.^{1, 2} The amino acid building blocks are naturally occurring, so there is potential for these materials to exert minimal toxic and immunogenic effects toward the host. The existence of many natural and synthetic residues

Supporting Information The Supporting Information includes individual vesicle molecular weight calculations, quantitative analysis of Tf-EL vesicle endocytosis, three-dimensional reconstructions of LSCM images, cytotoxicity of the endocytosis inhibitors, inhibition of control molecules known to follow certain endocytic pathways, quantitative analysis of the endocytosis inhibition studies, intracellular trafficking of the Tf molecule, intracellular trafficking of the Tf-EL vesicle at different time points, and intracellular trafficking of the Tf-EL vesicle in the presence of unconjugated Tf. This material is available free of charge via the Internet at <http://pubs.acs.org>.

of amino acids can also provide a wide range of chemical properties and biofunctionality. Recently, we developed a block copolypeptide, poly(L-arginine)₆₀-*b*-poly(L-leucine)₂₀ (R₆₀L₂₀), that forms spherical vesicles in aqueous solution.³ The presence of arginine on the surface of these vesicles enhanced their ability to be internalized by cells, as arginine-rich peptides are known to penetrate across the cell membrane.⁴ However, one disadvantage of these vesicles is their concentration dependent toxicity, which may limit their application as drug carriers since the vesicles alone at higher concentrations can be quite toxic. One potential application for the R₆₀L₂₀ vesicles would be to use them as carriers for DNA or siRNA, since the concentration of vesicles required for the delivery of these therapeutics is generally low, where studies have shown that gene expression can be manipulated with picomolar concentrations of genetic material.⁵⁻⁷ In order to be effective as a vehicle for other therapeutics such as doxorubicin (DOX) that require higher doses (micromolar *in vitro*), higher concentrations of the vesicles need to be administered as well.^{8, 9} Unfortunately, at these higher concentrations, the toxicity of the R₆₀L₂₀ vesicles alone can be quite significant, indicating that these vesicles would not be suitable for delivering these types of drugs.¹⁰

In addition to the R₆₀L₂₀ polypeptide, we previously reported an alternative polypeptide vesicle construct, poly(L-glutamate)₆₀-*b*-poly(L-leucine)₂₀ (E₆₀L₂₀) (Figure 1)¹¹. This polypeptide can also form spherical vesicles with sizes that can be controlled and can encapsulate hydrophilic molecules. Most importantly, these vesicles are less cytotoxic than the R₆₀L₂₀ vesicles due to their negative charge, as these vesicles do not have the detrimental effects associated with a positively charged drug carrier interacting with a net negatively charged cell membrane.¹⁰

However, one disadvantage of the E₆₀L₂₀ (EL) vesicles, compared to the R₆₀L₂₀ vesicles, is their inability to enter cells on their own. This disadvantage poses a limitation, since many therapeutics have intracellular targets. One approach to overcome this limitation is to conjugate a ligand that binds to a specific receptor overexpressed on the cell surface. In fact, transferrin (Tf) is a well-known ligand that binds to its receptor that is overexpressed on the surfaces of many cancer cells.¹² Tf is an iron-binding glycoprotein (~78,000 Da) that transports iron, an essential nutrient for cellular growth and proliferation, throughout the body. Iron is delivered via the transferrin receptor (TfR) through a receptor-mediated endocytosis pathway, specifically, a clathrin-mediated endocytosis pathway.¹³⁻¹⁵ Since cancer cells require more iron to sustain their rapid proliferation, TfR is overexpressed on the surfaces of many types of cancer cells.^{14, 15} Accordingly, numerous investigations have been performed using Tf as a cancer-targeting agent.^{12, 16}

In this study, Tf was conjugated onto the surface of the EL vesicles in order to give the vesicles the ability to target cancer cells and be internalized by them. The intracellular trafficking properties of the Tf-conjugated EL (Tf-EL) vesicles were evaluated using laser scanning confocal microscopy (LSCM) and immunostaining experiments.

Experimental Section

Materials

The PSCA-transfected 22Rv1 cell line was generated by retroviral gene transfer, as previously described.^{17, 18} The LAPC-4 cell line was kindly provided by Dr. Lily Wu (University of California, Los Angeles). Roswell Park Memorial Institute (RPMI) 1640 medium, Iscove's Modified Dulbecco's (IMDM), penicillin-streptomycin (P/S), sodium pyruvate (NaPyr), phosphate-buffered saline (PBS), and 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) were purchased from Invitrogen (Carlsbad, California). Fetal bovine serum (FBS) was obtained from Hyclone (Waltham, Massachusetts). Dialysis bags (MWCO = 1,000 Da) were purchased from Spectrum Laboratories (Rancho Dominguez, California). The formalin solution used to fix cells was obtained from Thermo Fisher Scientific (Waltham, Massachusetts), and the antibodies for the immunofluorescence experiments were products of Abcam (Cambridge, Massachusetts). Eight-well chambered coverglass units were obtained from Lab-Tek (Rochester, New York). The MTS cell proliferation assay kit was purchased from Promega (Madison, Wisconsin). The Bradford reagent was obtained from Bio-Rad (Hercules, California). Zeba desalt spin columns, N-hydroxysuccinimide (NHS), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Pierce (Rockford, Illinois). Spin concentrators (MWCO = 100,000 Da) were purchased from Millipore (Billerica, California). All other reagents, such as apo-transferrin (apo-Tf), were purchased from Sigma-Aldrich (St. Louis, Missouri) unless otherwise noted.

Cell Culture

The PSCA-transfected 22Rv1 cell line was grown in RPMI 1640 supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin at a pH of 7.4. The LAPC-4 cell line was grown in IMDM supplemented with 10% FBS, 1 mM sodium pyruvate, 100 units/mL penicillin, and 100 µg/mL streptomycin at a pH of 7.4. All cell lines were maintained in a 37°C humidified atmosphere with 5% CO₂ and handled with standard sterile tissue culture protocols.

Synthesis and Processing of EL Vesicles

The EL block copolypeptide was synthesized using the transition metal-mediated α -amino acid *N*-carboxyanhydride (NCA) polymerization technique, as described previously.¹⁹ The synthesized polymer was deprotected and dialyzed (MWCO = 1,000 Da) exhaustively against sterile Milli-Q water to remove any contaminants under pyrogen-free conditions. Fluorescein isothiocyanate (FITC) and ethylene diamine (ED) were conjugated together to form fluorescein-ethylene diamine (FITC-ED), which was later used to label the polypeptide for visualization and quantification purposes. Briefly, 2.6 mmol of ED was cooled to 0°C in 2 mL of DMSO. Subsequently, 0.026 mmol of FITC was dissolved in 2 mL of DMSO and added drop wise to the ED solution while stirring vigorously. The solution was sealed and covered with foil and allowed to react for 4 hrs at 0°C. To this solution, 2 mL of toluene were added, and the volatile compounds were removed by high vacuum. The addition of Toluene was repeated multiple times to ensure removal of all volatile compounds. The FITC-ED was subsequently conjugated to the EL polypeptide using EDC/NHS chemistry.

Briefly, 1% w/v EL polypeptide in 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer at a pH of 6.0 was prepared. EDC, NHS, and FITC-ED were added subsequently, such that the EL polypeptide:EDC:NHS:FITC-ED molar ratios were 4:10:1:1. The mixture was incubated overnight at room temperature. Afterwards, the solution containing the conjugated samples was purified through dialysis (MWCO = 1,000 Da) for 2 days, and the resulting samples were freeze-dried.

In order to form the FITC-labeled EL vesicles, equal volumes of 1% w/v FITC-labeled polypeptide and 1% w/v unlabeled polypeptide were separately prepared in tetrahydrofuran (THF) and then combined together to form a mixture with a 1:1 molar ratio of FITC-labeled to unlabeled polypeptide. Subsequently, an equal volume of sterile Milli-Q water was slowly added to yield a final polypeptide concentration of 0.5 % w/v. The mixture was dialyzed (MWCO = 1,000 Da) against sterile Milli-Q water overnight to remove the THF, where the water was changed every hr for the first 4 hrs. The large polydisperse vesicles were then extruded serially through 1000, 400, and 200 nm polycarbonate membranes to form vesicles with diameters of approximately 100 nm. The sizes of the vesicles and their distribution were analyzed with dynamic light scattering (DLS) measurements using the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, Massachusetts). The Bradford assay was performed to quantify the final concentration of polypeptide vesicles according to the manufacture-supplied instructions using the predialyzed samples as the standard.

Iron loading of Tf

Prior to all experiments, apo-Tf was iron loaded to make holo-Tf. Briefly, 20 μ L of the iron chelating agent nitrilotriacetate (NTA) was mixed with 10 μ L of 250 mM iron (III) chloride. An appropriate amount of this mixture was subsequently added to apo-Tf, dissolved in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing 20 mM sodium bicarbonate in a 10:1 iron:Tf molar ratio. The mixture containing iron and Tf was incubated at room temperature for 2 hrs to ensure all the Tf molecules were iron loaded. Excess free iron from the iron-loaded samples was removed with the Zeba desalt spin columns.

Conjugation of Tf ligands to EL Vesicles

In order to conjugate holo-Tf onto these vesicles, the carboxylate groups on the vesicle surfaces were activated using NHS and EDC. 10 mL of vesicles in sterile Milli-Q water (1 mg/mL) were mixed with NHS and EDC, using molar ratios of 5000:1 NHS:vesicle and 5000:1 EDC:vesicle. In order to achieve these ratios, the molecular weight of a single vesicle was estimated (Supporting Information) and used to quantify the number of vesicles in a given sample. The reaction proceeded for 25 min and then was quenched by adding 10x phosphate buffer (0.5 M) to raise the pH to 7.0. To conjugate Tf to the vesicle surface, holo-Tf (5000:1 Tf:vesicle molar ratio) was added to the suspension of activated vesicles. The solution was then stirred for 2 hrs at room temperature to form the Tf-EL vesicles. Afterwards, the conjugated vesicles were purified using a spin concentrator (MWCO = 100,000 Da) to remove the free Tf and EDC/NHS and to have them in a final buffer of 50 mM HEPES supplemented with 20 mM sodium bicarbonate.

Characterization of Tf-EL Vesicles

The sizes of the Tf-EL vesicles were determined with DLS measurements. The concentrations of the vesicles were obtained by measuring the fluorescence of the FITC conjugated to the vesicle and using a standard curve that was previously constructed with known concentrations of vesicles. The concentration of Tf was quantified with the bicinchoninic acid (BCA) protein assay, using a standard curve that was constructed with known concentrations of Tf in the presence of fixed amounts of vesicles.

Cytotoxicity Study with Tf-EL Vesicles

The CellTiter 96® AQueous Non-radioactive Cell Proliferation Assay (MTS assay) was used to determine cell viability. The cytotoxicity of the Tf-EL vesicles was evaluated using LAPC-4 and PSCA-transfected 22Rv1 cells. All cells were seeded onto each well of a 96-well tissue culture plate with a density of 8×10^4 cells/cm². EL and Tf-EL vesicles were prepared in growth medium, and their concentrations were varied from 20 to 100 µg/mL. After aspirating the original medium from each well, 100 µL of the prepared medium containing the vesicles was added to each well and incubated for 24 hrs in a humidified environment (37°C, 5.0% CO₂). Subsequently, 20 µL of the MTS reagent was added to each well, and the plate was incubated for an additional 1 hr. Cell viability relative to control wells (cells incubated in media without vesicles) was quantified by reading the visible light absorbance values at 490 nm and 700 nm with an Infinite F200 plate reader (Tecan System Inc., San Jose, California).

Laser Scanning Confocal Microscopy (LSCM)

LSCM images were taken on a Leica Inverted TCS-SP MP Spectral Confocal and Multiphoton Microscope (Heidelberg, Germany) equipped with an argon laser (488 nm blue excitation: JDS Uniphase), a diode laser (DPSS; 561 nm yellow-green excitation: Melles Griot), a helium-neon laser (633 nm red excitation), and a two photon laser setup consisting of a Spectra-Physics Millennia X 532 nm green diode pump laser and a Tsunami Ti-Sapphire picosecond pulsed infrared laser tuned at 768 nm for UV excitation. Images investigating the uptake of vesicles and their endocytosis mechanism were taken using an airy unit of 1.5 in order to project fluorescence signals through an entire cell; whereas, the images investigating the intracellular trafficking of vesicles were taken using an airy unit of one, where the goal is to capture fluorescence signals on a thin focus plane. This is to ensure that fluorescent signals that colocalize are all on the same plane of focus.

Uptake of Tf-EL Vesicles

LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units using a density of 8×10^4 cells/cm². FITC-labeled Tf-EL and EL vesicles were diluted in blank media to a concentration of 100 µg/mL. The cells were then incubated with the vesicles in serum-free media for 5 hrs in a 37°C humidified atmosphere with 5% CO₂. Following this incubation, the media was aspirated, and the cells were washed with PBS to remove any free-floating polypeptide vesicles that were not internalized before the confocal images were taken.

Investigating the Endocytosis Mechanism of Tf-EL Vesicles

To elucidate which endocytosis pathways play a role in the uptake of the Tf-EL vesicles, various drugs were used to block specific pathways, and the effect of each drug on the internalization of vesicles was observed. LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units at a density of 8×10^4 cells/cm². At the beginning of the experiment, the cell culture media was aspirated, and the cells were pretreated with various endocytosis inhibitors diluted in serum-free media at different concentrations for 30 min. Afterwards, the media containing the inhibitors was aspirated, and the cells were washed with PBS to remove any residual amount of inhibitors on the cell surface. The cells were then incubated in media containing 100 µg/mL of Tf-EL vesicles for 5 hrs. Afterwards, the cells were washed and visualized with the confocal microscope. In addition, the internalization behavior of the Tf-EL vesicle was investigated in the presence of excess Tf. In this case, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated in medium containing both Tf-EL vesicles (100 µg/mL) and an excess of free Tf molecules (100 times the concentration of conjugated-Tf in media).

Intracellular Trafficking of Tf-EL Vesicles

Immunostaining of cells was performed to identify whether the Tf-EL vesicles were localized with the early endosome or lysosome after internalization. LAPC-4 and PSCA-transfected 22Rv1 cells were seeded onto eight-well chambered coverglass units using a density of 8×10^4 cells/cm². In the experiments which involved the staining of the early endosomes, cells were incubated in serum-free media containing Tf-EL vesicles (100 µg/mL) for 5 hrs at 16°C instead of 37°C. The purpose of lowering the incubation temperature from 37°C to 16°C was to aid visualization by decreasing the rate at which the Tf-EL vesicles were recycled, since this decrease in temperature has been used when studying the Tf ligand itself.²⁰ At the end of this incubation period, the vesicles were removed, and the cells were briefly washed with PBS to remove any vesicles on the cell surface. The cells were subsequently fixed with a 10% formalin solution for 10 min at room temperature and washed three times with PBS, where each wash lasted 5 min. After fixation, the cells were permeabilized with a 0.25% Triton X-100 solution for 10 min, followed by three 5 min PBS washes. Nonspecific antibody binding sites on and in the cells were then blocked by incubating the fixed and permeabilized cells with a blocking solution containing 5% nonfat milk in PBS for 30 min. Rabbit polyclonal primary antibodies specific for human early endosome antigen-1 (EEA-1) were then added at a 1:500 dilution in blocking solution and incubated overnight at 4°C. Cells were then washed three times in PBS, where each wash lasted 10 min. These washes were followed by incubation with goat polyclonal antibodies against rabbit IgG conjugated with Cy5. The secondary antibodies were added at a 1:500 dilution in the blocking solution and incubated for 1 hr at room temperature, followed by three more 10 min PBS washes. At the last step, the cells were incubated for 5 min with a 300 nM DAPI solution, followed by three 5 min PBS washes before the LSCM images were taken. Each image consisted of three channels: green for Tf-EL vesicles, blue for cell nucleus, and red for the labeled intracellular compartments (either the early endosomes or lysosomes). In order to perform the experiments that involved the staining of lysosomes, the above procedure was repeated except for the use of primary antibodies specific for the lysosomal-associated membrane protein-1 (LAMP-1) to stain the lysosomes.

Results and Discussion

After conjugating Tf to the EL vesicles with an incubation ratio of 5000 Tf per vesicle, the size and polydispersity index (PDI) of the conjugated vesicles were measured using DLS. The average diameter of the vesicles was 121 ± 6 nm with an average PDI of 0.243 ± 0.056 , which is within the size range that could take advantage of the enhanced permeability and retention (EPR) effect.^{21, 22} Fluorescence measurements of the FITC-labeled vesicles and the BCA protein assay were used to quantify the vesicle and Tf concentrations, respectively. These values were subsequently used to quantify the number of Tf conjugated per vesicle, which was approximately 1930 ± 190 , representing an average conjugation yield of 38%.

The *in vitro* cytotoxicity of the Tf-EL vesicles was assessed using the prostate cancer cell lines and the MTS assay. Figure 2 shows that the Tf-conjugated EL vesicles had no toxic effect on the LAPC-4 and PSCA-transfected 22Rv1 cells at concentrations ranging from 40 to 200 $\mu\text{g/mL}$, as the relative survival of the cells incubated with the vesicles was approximately 100%. This was not surprising since the EL vesicles and Tf molecules are individually nontoxic, and therefore, the conjugate formed from the two should also be nontoxic.

In order to investigate whether the addition of Tf would increase the cellular uptake of the E₆₀L₂₀ vesicles, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated with the FITC-labeled Tf-EL and EL vesicles, and the level of fluorescence inside the cells was visualized using confocal microscopy. For both cell lines, a low level of fluorescence inside the cell was observed when the cells were incubated with the non-targeted EL vesicles, indicating minimal vesicular uptake (Figures 3a and c). However, when Tf was conjugated to the FITC-labeled vesicles, the level of fluorescence was significantly enhanced, implying greater vesicle uptake and improved targeting towards cancer cells (Figures 3b and d). This uptake was quantified using flow cytometry and ImageJ analysis, and the quantitative results also demonstrated that the conjugated Tf can indeed improve the internalization by several fold (Figures S2 and S3 in the Supporting Information). The three-dimensional reconstructions of the LSCM images were also obtained and confirmed that the vesicles are indeed internalized within the interior of the cells (Figure S4 in Supporting Information).

Once we confirmed the increased uptake of vesicles due to Tf, we sought to determine the mechanism of internalization for these vesicles. Identifying this property can help us determine where the vesicles traffic after internalization, as different mechanisms of endocytosis affect intracellular fates differently. There are three well-studied mechanisms by which molecules are endocytosed by cells: macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis. Macropinocytosis is a nonspecific mechanism for internalizing molecules in the extracellular environment via the formation of membrane ruffles, where the internalized macropinosomes can have sizes up to 5 μm in diameter.²³ Clathrin-mediated endocytosis is a specific type of receptor-mediated endocytosis, where molecules, such as Tf, are taken up by clathrin-coated vesicles, which have diameters that range from 150 to 200 nm.^{24, 25} Caveolae-mediated endocytosis involves the formation of vesicles in the lipid raft region of the plasma membrane in a caveolae-dependent manner.

The internalized vesicle from the caveolae is the smallest among the three with a diameter between 50 and 80 nm.²⁶

To determine the mechanism of cellular uptake for the Tf-EL vesicles, we used drugs that could inhibit each specific pathway, and observed their effects on the endocytosis of the vesicles.²⁷ Specifically, LAPC-4 or PSCA-transfected 22Rv1 cells were separately treated with cytochalasin D and amiloride to observe the effect of inhibiting macropinocytosis. In addition, chlorpromazine and dynasore were used to inhibit clathrin-mediated endocytosis, and nystatin and filipin III were used to inhibit caveolae-mediated endocytosis. Initially, the toxic effects of the drugs were investigated with both cell lines, since cytotoxic effects are known to increase membrane permeability, which could confound our endocytosis results.²⁸ For both cell lines, a range of concentrations for each drug was investigated, and the maximum concentration that exhibited at least 80% survival was chosen for the endocytosis inhibition experiments (Figures S5-S10 in Supporting Information). We also confirmed that the drugs at these concentrations were able to inhibit the intended pathways by testing the inhibitors with molecules known to follow certain endocytic pathways (Figures S11 and S12 in the Supporting Information).

After determining drug concentrations that were minimally toxic to the cells, the effect of each drug on the uptake of the vesicles was investigated. Incubating LAPC-4 cells with the Tf-EL vesicles without the presence of an inhibitor leads to a high level of vesicle uptake as shown in Figure 4a. Pretreating the cells with inhibitors of clathrin-mediated endocytosis, namely, chlorpromazine and dynasore, had the most dramatic effect on inhibiting the vesicle's uptake (Figures 4b and c). In addition, when cells were incubated with an excess amount of free Tf, which enters cells through clathrin-mediated endocytosis, the Tf-EL vesicle's ability to be endocytosed also decreased, indicating that the excess Tf was able to outcompete the Tf on the vesicles for receptor binding and effectively inhibited the endocytosis of the vesicles (Figure 4d). The effects of inhibiting the other pathways, such as macropinocytosis and caveolae-mediated endocytosis, were also investigated. Treatment with macropinocytosis inhibitors, such as cytochalasin D and amiloride, led to some level of inhibition, but not as significant as the inhibition observed with inhibitors of clathrin-mediated endocytosis (Figures 4e and f). Finally, inhibitors of caveolae-mediated endocytosis, namely, nystatin and filipin III, were also tested. In this case, there was minimal reduction in uptake due to these caveolae-mediated endocytosis inhibitors (Figures 4g and h). Based on these results, we concluded that clathrin-mediated endocytosis dominates the endocytosis pathway of the Tf-EL vesicles, which implies that the binding of Tf on the vesicles to Tf receptors on the cell surface is responsible for the internalization, since the Tf ligand itself enters cells via this mechanism. It is plausible that macropinocytosis can contribute to the vesicle internalization as well, where membrane ruffles can nonspecifically internalize the Tf-vesicles that are bound to the cell surface through Tf/TfR interactions. To test whether this phenomenon is cell-line dependent, the uptake inhibition experiments were repeated on the PSCA-transfected 22Rv1 cell line (Figures 4i-p). The inhibition results were consistent with those from the LAPC-4 cells, suggesting that the endocytosis behavior of the Tf-EL vesicle is not dependent on the cell-line. Further analysis using ImageJ quantitatively supported the trends shown in Figure 4 (Figure S13 in the Supporting Information Section).

Molecules and nanoparticles internalized via clathrin-mediated endocytosis and macropinocytosis are generally first trafficked to early endosomes, where they are typically either sorted to lysosomes for degradation or are recycled back to the cell surface.²⁹ Moreover, the Tf ligand is well known for being trafficked to early endosomes and then recycled out of the cell.³⁰⁻³² Accordingly, we expected a similar intracellular fate for our Tf-EL vesicles, and immunostaining experiments were performed with cells after they internalized the Tf-EL vesicles to investigate the vesicle trafficking behavior. Initially, LAPC-4 and PSCA-transfected 22Rv1 cells were incubated with the Tf-EL vesicles for 5 hrs at 37°C, followed by immunostaining and confocal microscopy to visualize the early endosomes, which were stained using EEA-1 (Figures 5a and e). Results from this initial study indicated that the vesicles did not colocalize with the early endosomes, which was unexpected as molecules endocytosed by clathrin-mediated endocytosis and macropinocytosis are generally routed to early endosomes. However, reports from other research groups have shown that the Tf ligand recycles very quickly (~5 min), making it difficult to maintain and observe Tf in the early endosomes during the washing steps preceding fixing of the cells for imaging.^{20, 33} These same research groups also reported that, by lowering the incubation temperature and the temperature of the wash steps to 16°C, the recycling of the Tf ligand out of the early endosomes was decreased, allowing significantly more Tf to remain in the early endosomes immediately prior to fixing the cells. After we confirmed that we could observe the Tf ligand itself colocalized with early endosomes at this lower temperature (Figure S14 in Supporting Information), we incubated FITC-labeled Tf-EL vesicles with LAPC-4 and PSCA-transfected 22Rv1 cells at 16°C, and the results of the early endosome immunostaining using EEA-1 are shown in Figures 5c and g. Colocalization between the vesicles and the EEA-1 antibody are represented by yellow or orange fluorescence, since the fluorescent tag on the vesicle is FITC (green), and the fluorescent label on the secondary antibody for the primary antibody for EEA-1 is Cy5 (red). The figures show that there is significant colocalization of Tf-EL vesicles with the early endosomes. These results indicate that the vesicles are routed to the early endosomes, which is consistent with the behavior of other molecules and nanoparticles that are internalized via clathrin-mediated endocytosis and macropinocytosis.

To determine if the vesicles are routed from the endosomes to the lysosomes for degradation, LAPC-4 and PSCA-transfected 22Rv1 cells were again incubated with FITC-labeled Tf-EL vesicles at temperatures of 37°C and 16°C, followed by immunostaining for LAMP-1 (Figures 5b, d, f, and h). There was no colocalization observed between the green fluorescence signal from the vesicles and the red fluorescence signal from the LAMP-1 immunostaining in either cell line for both temperatures. These results suggest that the Tf-EL vesicles do not traffic to the lysosomes, but probably recycle back to the cell surface. Note that we also investigated the intracellular trafficking of the vesicles using shorter incubation times of 30 min and 2.5 hr (Figures S15 and S16 in the Supporting Information); however, we found the 5 hr incubation time point yielded the highest amount of internalized vesicles, which allowed the highest possibility for colocalization. Lastly, to further confirm the recycling of our vesicles, we coincubated our cells with the Tf-EL vesicles and unconjugated Tf, and observed significant colocalization between them, indicating that they are present in the same intracellular compartments and are undergoing the same intracellular

fate (Figure S17 in the Supporting Information). This same recycling fate was expected since de Witte and coworkers used fluorescence uptake experiments to demonstrate that their Tf-conjugated polyethylene glycol (PEG)-liposomes exhibited similar localization and trafficking patterns as the Tf ligand.³⁴ Based on our findings, we expect drugs encapsulated in the Tf-EL vesicles to be routed to the early endosomes, and then recycled back to the cell surface, which could prevent the release of the drugs inside the cell. Hence, a mechanism for disrupting the endosome could potentially allow the vesicle to avoid recycling and enhance their time inside cells.

Conclusions

Stable and permanent conjugation between Tf and the E₆₀L₂₀ vesicles allows the formation of nontoxic Tf-conjugated EL vesicles that enhance the cellular uptake of these negatively charged nanocarriers. Subsequent endocytosis and immunostaining studies have shown that the presence of transferrin on the vesicles affects their internalization and intracellular trafficking properties. Clathrin-mediated endocytosis, the pathway by which the Tf ligand is internalized by cells, was identified as the dominant mechanism by which these vesicles enter cells with a smaller contribution from the macropinocytosis pathway. The Tf-EL vesicles were also found to traffic to the early endosomes but not the lysosomes, suggesting that the vesicles are recycled back to the cell surface. From these results, a design criterion emerges, where incorporating an endosomal disrupting property to these vesicles could allow them to break out of the endosomes and prevent their recycling, thereby increasing their localization within the cytosol.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Schematic of the E₆₀L₂₀ block copolypeptide and vesicle assembly.

Figure 2.

The results of 24 hr cytotoxicity experiments for the Tf-EL vesicles incubated with LAPC-4 (red) and PSCA-transfected 22Rv1 (blue) cells. Error bars represent the standard deviation from an average of three measurements.

Figure 3.

LSCM and corresponding differential interference contrast (DIC) images of LAPC-4 cells incubated with 100 $\mu\text{g/mL}$ of either (a) non-targeted FITC-labeled EL vesicles or (b) FITC-labeled Tf-EL vesicles for 5 hrs in serum-free media. PSCA-transfected 22Rv1 cells were also incubated with either (c) non-targeted FITC-labeled EL vesicles or (d) FITC-labeled Tf-EL vesicles. Scale bar = 30 μm .

Figure 4.

Inhibition of cellular uptake of the Tf-EL polypeptide vesicles (green) using inhibitors prior to incubation with the vesicles. LSCM and corresponding DIC images are shown for each condition. (a) LAPC-4 control cells with no inhibitor pretreatment. For the inhibition of clathrin-mediated endocytosis, LAPC-4 cells were pretreated with either (b) chlorpromazine or (c) dynasore, or simultaneously treated with (d) excess free Tf (100 times the concentration of conjugated-Tf in media) along with the vesicles. For the inhibition of macropinocytosis, LAPC-4 cells were pretreated with either (e) cytochalasin D or (f) amiloride. For the inhibition of caveolae-mediated endocytosis, LAPC-4 cells were treated with either (g) nystatin or (h) filipin III. The inhibition experiments were repeated with the PSCA-transfected 22Rv1 cells, where the cells were pretreated or simultaneously treated either with (i) media only, (j) chlorpromazine, (k) dynasore, (l) excess free Tf, (m) cytochalasin D, (n) amiloride, (o) nystatin, or (p) filipin III. Scale bar = 30 μ m.

Figure 5.

Colocalization of Tf-EL vesicles with intracellular compartments. LAPC-4 cells were incubated with Tf-EL vesicles (green) at 37°C for 5 hrs, and stained either with (a) EEA-1 (red) or (b) LAMP-1 (red). The same cells were also incubated with the vesicles at 16°C for 5 hrs, and stained with either (c) EEA-1 or (d) LAMP-1. PSCA-transfected 22Rv1 cells were incubated with Tf-EL vesicles at 37°C for 5 hrs, and stained either with (e) EEA-1 or (f) LAMP-1. The same cells were also incubated with the vesicles at 16°C for 5 hrs, and stained with either (g) EEA-1 or (h) LAMP-1. The nucleus for each cell was stained with DAPI (blue). Scale bar = 20 μ m.