Lysosome-Targeting Strategy Using Polypeptides and Chimeric Molecules

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ABSTRACT: Lysosomes are membranous compartments containing hydrolytic enzymes, where cellular degradation of proteins and enzymes among others occurs in a controlled manner. Lysosomal dysfunction results in various pathological situations, such as several lysosomal storage disorders, neurodegeneration, infectious diseases, cancers, and aging. In this review, we have discussed different strategies for synthesizing peptides/chimeric molecules, their lysosome-targeting ability, and their ability to treat several lysosomal associated diseases, including lysosomal storage diseases and cancers. We have also discussed the delivery of cargo molecules into the lysosome using lysosome-targeting ligand-decorated nanocarriers. The introduction of a protein-binding ligand along with a lysosome-targeting ligand to manufacture a chimeric architecture for cell-specific protein (extracellular and membrane protein) degradation ability has been discussed thoroughly. Finally, the future applications of these lysosome-targeting peptides, nanocarriers, and chimeric molecules have been pointed out.

LYSOSOME AND ITS FUNCTION

Lysosomes are membrane-bound acidic cellular organelles containing hydrolytic enzymes that degrade various biomacromolecules such as carbohydrates, nucleic acids, proteins, fats, and cellular components.1,2 Belgian biologist Christian de Duve discovered these organelles, a part of the endomembrane system, and coined the term “lysosome,” for which he was awarded the Nobel Prize in Medicine in 1974.1 This acidic environment (pH ~ 4.5−5.0) is maintained by a vacuolar ATPase that actively pumps protons in-between the cytoplasm and lysosome.3 Highly glycosylated lysosome-associated membrane proteins Lamp-1 and Lamp-2 protect the internal lysosomal environment from the cytoplasmic environment. These lysosomal membrane components play diverse and crucial roles in lysosome homeostasis.7 Apart from the degradation of biomacromolecules and biogenesis, the primary function of the lysosome is controlling cellular responses to nutrients. A lysosomal membrane kinase protein complex, mammalian target of rapamycin complex 1 (mTORC1), regulates cellular responses such as nutrient/energy/redox sensing and controls protein synthesis inside the cell.4,5 Lysosomes are spatially linked with mTOR autophagy-dependent protein degradation and recycling, allowing new building blocks to maintain several cellular functions.6 The role of lysosomes and autophagy appears to be related to programmed cell death.6 Thus, lysosomes play different roles during development and differentiation, detecting morphogen gradients, remodeling intracellular components during the cell differentiation, and participating in cell demise, either by directly inducing cathepsin-dependent cell death or degrading apoptotic cells.7,8 All the above evidence suggests that lysosomes are essential and active players in controlling several cellular responses to nutritional stress.

LYSOSOMAL DYSFUNCTION

The cell types and their environment influence the functioning of the lysosome. The change of any lysosomal function causes several disorders, including neurodegenerative disorders, cancers, and metabolic disorders. Lysosomes protect any damage to the cells from immune regulation.8 However, genetic defects, environmental factors, and deficiency of any one enzyme in the lysosomes may impair its function, and as a result, accumulation of the substrates occurs, causing widespread harm to cells.8 Metabolic machinery may be impaired, and other cell organelles such as mitochondria and peroxisomes may be dysfunctional. Lysosomal Storage Disorder (LSD) is an example of lysosomal dysfunction (Table 1).9−11 The complete absence or insufficiency of any

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| Disease | Lysosomal Dysfunction (Deficiency in the Enzyme) | Observations/Comments | References |
|---------|--------------------------------------------------|-----------------------|------------|
| LSD | Aspartylglucosaminidase | Progressive mental health problems with skeletal and connective tissue abnormalities in humans | 11 |
| Fabry disease | α-Galactosidase | Inherited LSD characterized by immune deficiency (susceptibility to infections including pulmonary infections), cardiac, cerebral, and renal diseases in patients | 12 |
| Gaucher disease (types 1, 2 and 3) | β-GCase | Abnormalities in the visceral organs (type 1) and neurological defects in both children and adults (types 2 and 3) | 12 |
| Mucopoly saccharidosis (I, II, III, IV, VI, VII) | α-L-Iduronidase, Iduronate 2-sulfatase, Heparan N-sulfatase, Galactose 6-sulfatase, N-acetylgalactosamine 4-sulfatase, and β-glucuronidase | Coarse facial features (including a flat nasal bridge, thick lips, and enlarged mouth and tongue), dwarfism, abnormal bone size and/or shape (dysplasia), thickened skin, enlarged organs such as liver or spleen | 7, 11 |
| GM1 gangliosidosis | β-Galactosidase | Skeletal manifestations and neurological impairment in humans | 11 |
| Krabbe disease (globoid cell leukodystrophy) | Galactocerebrosidase | Brain histology shows myelin loss, neuroinflammation, and axonal degeneration | 13 |
| Pompe disease | α-Glucosidase | Accumulated undegraded glycogen in the muscles and peripheral nerves was observed in humans | 11 |
| Sandhoff disease (type II and III) | β-Hexosaminidase A and B | Causes motor function and neurological disorders in humans | 11 |
| Danon disease | LAMP2 | Cause accumulation of glycogen and other autophagic components in the cardiomyocytes of humans, which results in cardiac diseases | 15 |
| Crohn’s disease | Abnormal lysosomal pH | Deregulation of proton-sensing G protein-coupled receptor (GPR65) was observed in both mice and humans | 16 |
| Rheumatoid arthritis | Lysosomal hydrolases | In humans, different cathepsins, acid phosphatases, and others are involved in the inflammation and joint damage | 17 |
| Multiple sclerosis | Lysosomal acidification | Defects in the lysosomal compartment lead to defects in lipid droplet degradation in human neuronal cells | 18 |
| Alzheimer disease | Unbalanced lysosomal luminal pH | In humans, defective presenilin-1 dependent v-ATPase function was observed in the case of lysosomal acidification. Lysosomal non-specific cathepsins generate the β-amyloid protein and hyperphosphorylated tau proteins | 19 |
| Parkinson disease | Alterations in the lysosomal | Selective loss of GCase in lysosomes relates to the decreased amount of LAMP2A and increased cathepsins A and D in humans | 11 |
| CMA pathway | Mutation in Huntington (HTT) | Aggregate-prone protein HTT forms abnormal, toxic polyglutamine expansions, inhibition of autophagosome biogenesis, and transport | 20 |
lysosomal enzyme may cause severe metabolic disorders leading to immunodeficiency, and permanent LSD may lead to multiple organ damage, severe neurodegenerative disorders, Alzheimer’s disease, and many more.9,10 Intralysosomal accumulations of undigested materials due to lysosomal enzyme deficiency lead to LSD. To date, lysosomal dysfunctions have been associated with more than 50 monogenic human genetic diseases.9 These diseases are inherited and observed chiefly in utero or during early childhood, while it becomes milder in juveniles or adults. The consequences of LSDs altered several functions such as lipid trafficking, calcium homeostasis, and inflammation, which contribute to pathogenesis.9 Autophagosome accumulation and defects in autophagy lead to several neurodegenerative disorders in many LSDs. Several defective lysosomal functions have been pointed out between Alzheimer’s disease (AD) and defective lysosomal proteolysis. The translocation of the lysosomal v-ATPase V0a1 subunit is associated with the presenilin 1 protein, which undergoes mutation in AD. The reduction of lysosomal ATPase and presenilin 1 hampered the degradation of autophagic/lysosomal entities. Together, all these findings demonstrate that the proper lysosomal function is essential for the prevention of neurodegenerative disorders.9,10

In Parkinson’s disease (PD), the reduction in lysosomal protein (Lamp-1 and Lamp-2) level induces autophagosome accumulation and dopaminergic neuron cell death. Lysosomal dysfunction results in abnormal lysosomal membrane permeabilization (LMP) by reactive oxygen species produced from lysosomal-induced mitochondria. LMP-induced neurodegeneration is directly associated with lysosomal breakdown and autophagosome accumulation, resulting from lysosomal proteases released into the cell cytoplasm. Thus, the PDs can be treated using a novel neuroprotective strategy, where lysosomal enzyme levels and lysosomal functions can be restored.11 Alpha-synuclein undergoes degradation via the lysosomal pathway, and its accumulation is a feature of the PD and synucleinopathies, a type of neurodegenerative disease (family of LSD).7,11 The biochemical analysis described that the deficiency of the lysosomal enzyme glucocerebrosidase enhances the levels of soluble α-synuclein oligomers. Thus, aggregation-dependent neurotoxicity was observed, which is defined as Gaucher disease.14 Lafora disease is another LSD family’s neurodegenerative genetic disorder, where accumulations of insoluble abnormal glycogen (called polyglucosans) are observed in Lafora bodies, within the neurons and cells of the liver, heart, and skin.7,11 The glycogen undergoes degradation inside the lysosomes via an autophagy—lysosomal system and is catalyzed by acid alpha-glucosidase (known as maltase). This autophagy—lysosomal pathway is vital for glycogen degradation, and the lack of these single lysosomal enzymes resulted in glycogen storage disease type II, such as Pompe disease.8,11 All these findings demonstrate that lysosomal dysfunction appears as a common hallmark under various pathological conditions, including LSD, neurodegeneration, and aging.7

### LYSOSOME IN LMP-MEDIATED LYSOSOMAL CELL DEATH

Any damage into the lysosomal membrane that induces the release of the lysosomal contents into the cytoplasm results in indiscriminate degradation of cellular components such as enzymes.7 Additionally, massive lysosomal membrane damage may increase the cytosolic acidity that results in cell death by necrosis. The LMP-based cell death has drawn attention in the development of cancer treatments. Many agents can efficiently kill the cancer cells in vitro but fail to act in vivo due to their inefficient targeting ability.16,21,22 All cells have lysosomes, but the properties of cancer cells’ lysosomes are different, including size, intracellular localization, enzyme activity, and cathepsin expression from normal cells. These properties can be exploited as a suitable target for cancer treatments. Several studies found that the enlarged size of the cancer cells’ lysosomes render them more susceptible to LMP. Also, lysosome-dependent genetic mutations contribute to tumor development and the progression of genetic disorders. The autophagy induction and LMP-mediated (lysosomal destabilization by lysosomotropic agents like chloroquine) cell killing show remarkable antitumoral effects in vivo. Thus, LMP-mediated cell killing (lysosomal cell death) has become a new and highly potential strategy for cancer treatment in human tumors.23,24

### LYSOPHAGY OR REPAIR OF DAMAGED LYSOSOMES

Although LMP-mediated cell killing is a powerful tool for several disease treatments such as cancer and neurodegenerative diseases, in this strategy, many lysosomes remain slightly damaged and unstable, which impose several cellular stresses (due to lysosomal membrane destabilization). Thus, repairing those damaged and frail lysosomes or throwing them out from the cell is essential.25,26 The inability of the endosomal sorting complex required for transport (ESCRT) machinery, which repairs damaged lysosomes, induces the ubiquitin tagging on the irreversibly damaged lysosomes and initiates the clearance via selective macroautophagy, called lysophagy.27 Lysophagy and damaged lysosome repair are critical for several diseases, including cancer, aging, and neurodegenerative diseases.28 Thus, the development of new molecules, including small molecules and polymers, is highly important to target damaged lysosomes specifically, which can induce the quick clearance or repair of the damaged lysosomes.

### LYSOSOME-ASSOCIATED DISEASE TREATMENT STRATEGIES

Limited treatments are available for LSD diseases, and the lower number of LSD cases attracts difficulty in developing effective treatments.7 Many of these pathologies’ biological and medical complexity have rendered a limited number of treatments clinically available. Organ transplantation is the most classical therapeutic strategy, including liver transplantation, an organ primarily affected in most LSDs, and bone marrow transplantation. However, these strategies become ineffective for several LSDs. Another approach to treat LSDs is implantation or transplantation of healthy cells that express wild-type or regular copies of a given enzyme and reduce the deficiency of the same enzyme, thus correcting the defect. The lack of compatible donors reduces the availability of these implantation or transplantation strategies for LSD treatment. Recently, gene therapy has become a powerful technique for treating LSDs that provide relevant corrections in the enzyme deficiency in the peripheral organs such as the liver, but these technologies are inefficient to other tissues, such as the central nervous system (CNS). These therapeutic strategies can overcome several life-threatening damage
directly affecting tissues or their derived counterparts. Also, the average lysosomal enzyme production from healthy cells can theoretically correct several defects in the neighboring tissue via a process called “cross-correction.” This cross-correction phenomenon represents a significant pillar for the design of several therapeutic strategies. Before developing the LSD treatment strategies, we need to know the lysosomal pathway for lysosomal enzyme delivery.10−20

### SMALL MOLECULE THERAPY

A new strategy based on small molecules has the potential to overcome the limitations of enzyme replacement therapy (ERT) and is currently the cornerstone in the treatment of inherited lysosomal storage diseases (LSDs). Small molecule (Figure 1) therapy includes substrate reduction therapy (SRT), pharmacological chaperone therapy (PCT), and proteostasis regulators (PR), where SRT slows down the production of glycosphingolipids (GSL) in the lysosome, and the small number of glycosphingolipids (GSL) substrate degrades by residual lysosomal enzymes. Miglustat is a licensed small molecule drug to treat Gaucher disease and Niemann Pick disease type C LSDs. In contrast, Eliglustat and genistein are currently in clinical trials for Gaucher disease and Mucopolysaccharidoses diseases.11 In PCTs, chaperones stabilize the conformation of mutated lysosomal enzymes, increasing an active lysosomal enzyme in the lysosome. Migalastat is currently investigated in Fabry patients carrying missense mutations. In PRs, small molecules induce the misfolding of lysosomal enzymes by manipulating the endogenous chaperone machinery and signaling pathways, for example, calcium channel-signaling pathways. The major limitation of this small molecule therapy is off-target actions, which increase unwarranted side effects. Enhancement of target efficiency requires the development of nanomaterials, polymers, and peptides that can efficiently deliver these therapeutics.

### ENZYME REPLACEMENT THERAPY

ERT is a powerful therapeutic strategy for the treatment of LSDs that surmount the metabolic defects in patients by periodic intravenous administration of a functional recombinant version of the defective enzyme in the particular LSD. Typically, the enzyme is delivered to the target cells via mannose, or mannose-6-phosphate receptors (M6PR)-mediated endocytosis, which upon successful delivery degrades the accumulated substrates in the lysosomes to ameliorate LSD. The first successful and commercial biological therapy for the treatment of LSD is ERT. Several LSDs, such as type I Gaucher disease, Fabry disease, Pompe disease, Mucopolysaccharidosis (MPS) I, MPS II, and MPS VI, are treated employing ERT with recombinant enzymes, and many more ERTs have reached clinical trials to find the cure of several

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**Figure 1.** Small molecule for lysosomal disease treatment.
other LSDs. The main challenge of ERT is the recombinant expression of the enzymes with the M6P tag on them and it costs around 2,00,000$ annually, which makes ERT an expensive treatment. Blood–brain barrier crossing of intravenously administered recombinant enzymes and low expression of requisite cell surface receptors limit ERT for some LSDs, including CNS-manifested LSDs. Numerous developments focus on enzyme modification and nanocarriers/polymer/polypeptides for efficient enzyme delivery via intracerebroventricular/intrathecal administration.

### IMPORTANCE OF M6PRS IN DELIVERING PROTEINS/ENZYMES/ThERAPEUTICS TO THE LYsOSOME

The endogenous endoplasmic reticulum (ER)-secreted lysosomal enzymes are generally transported to the lysosomal compartment via the Golgi apparatus. In this transportation pathway, lysosomal enzymes containing mannose residues are additionally phosphorylated in the cis-Golgi network to form mannose-6-phosphate (M6P)-tagged lysosomal enzymes. In mammalian cells, the M6PRs play a crucial role in sorting and delivering lysosomal enzymes into lysosomes (Figure 2). These receptors are divided into two major parts: the ~300 kDa insulin-like growth factor-II (IGF-II)/cation-independent (CI) multifunctional transmembrane glycoprotein M6P receptor (CI-M6PR) and the ~46 kDa cation-dependent (CD) M6P receptors (CD-M6PR) (Figure 2). The cation-independent M6PRs (CI-M6PRs) are present in the trans-Golgi network (TGN), which binds with the M6P-tagged lysosomal enzymes (from the cis-Golgi network) and then transported into the acidic lysosome via a receptor and clathrin-mediated endocytosis. Furthermore, the overexpression of CI-M6PR receptors can serve as an early marker for several cancers, particularly in breast cancers, pancreatic cancer, gastric cancer, melanoma, and prostate cancer.

It has been demonstrated that M6PR may act as a potential tumor suppressor in 70% of hepatocarcinoma. The M6PR overexpression induces growth inhibition of cancer cells and regression of tumors in mice. Thus, M6PR appears to be a promising cancer prognostic marker and becomes a possible target for several therapies. Also, it is widely thought that M6PR prevents tumor progression by internalization and subsequent degradation of the mutagenic IGF-II, which is responsible for several cancers. In this way, M6PR can accelerate and suppress several diseases. The role of M6PR in cellular growth suppression is well studied, but its impact on tumorigenicity, invasiveness, and metastasis development is not completely understood, thus further studies are necessary. The potentiality of CI-M6PR for the cargo shuttling into the lysosome can thus be a specific target for M6P-labeled carriers to dispatch cytotoxic drugs and enzymes inside lysosomes for the treatment of several cancers and LSDs.

To accelerate the treatment of several lysosomal-dependent cancers and LSDs, developing a lysosome-specific ligand is highly important. Several studies describe that the cell surface receptors interact with the ligands differently, viz., monovalently with monomeric and polyvalently with polymeric ligands. The CI-M6PR exists as a dimer in the extracellular membrane and binds to the multivalent M6P-ligands with a high affinity ($K_d = 2–20$ nM) in comparison to monomeric M6P-ligands ($K_d = 7$ μM), defined as the “glycoside cluster effect.” The selectivity of M6P-ligands and the many-fold improvement of the binding affinity toward the CI-M6PR receptor has encouraged the scientific community to develop multiple M6P-ligands containing synthetic polymers to facilitate more effective lysosomal targeting.

### DESIGN STRATEGIES

Synthetic polymers need few attributes to target the lysosome efficiently (Figure 3). Such a design would include (i) polyvalent display of ligands that are known to traffic the polymer exclusively to the lysosome, (ii) ligands should be

![Figure 2. The structure of M6PRs. The two M6PRs are transmembrane glycoproteins and the only members of the p-type lectin family. The CD-M6PR (a) appears to be a homodimer at the membrane and it consists of an N-terminal extracytoplasmic region, a transmembrane domain, and a C-terminal cytoplasmic region. (b) CI-M6PR is also called M6P/IGF2-R because it binds both M6P ligands and IGF2. CI-M6PR seems to behave as a homodimer in the membrane. It contains a large N-terminal extracytoplasmic domain organized in 15 repeats, a short transmembrane region, and a small intracellular C-terminal domain.](https://doi.org/10.1021/acsomega.1c04771)

![Figure 3. Schematic representation of lysosome targeting using a lysosome-specific ligand-containing polymer.](https://doi.org/10.1021/acsomega.1c04771)
attached to the backbone via a flexible linker so that it could have the optimal binding affinity, (iii) introduction of suitable functional groups in the polymer side chain/end that would allow covalent attachment of drugs and bioproducts, (iv) the polymer backbone should be prone to degradation inside the lysosome, and (v) the affinity of the ligand to cell surface-receptors for efficient endocytosis. Several studies have indicated that ligands such as M6P, mannose-6-phosphonate (M6Pn), and tri-GalNAC show efficient trafficking toward the lysosome and are exclusively internalized via a receptor (CI-M6PR and ASGP-R)–mediated endocytosis.

Keeping these attributes in mind, polypeptides having the M6P ligand on the side chain were synthesized. Previous studies have shown that the ligand M6P is internalized via the C1-M6PR receptor and then exclusively trafficked into the lysosome. Hence, the development of M6P-based glycopolypeptide that would polyvalently display M6P from a biodegradable polypeptide backbone has been an essential target in this endeavor.

**LYSOSOME-TARGETING M6P-LIGAND-BASED OLGEOPEPTIDE**

In 2014, Overkleeft et al. developed a synthetic multivalent M6P-ligand-containing oligomer for the trafficking of the endolysosomal pathway via the C1-M6PR receptor targeting (Figure 4). To prove the potential M6PR-targeting proper-

![Figure 4](https://example.com/figure4.png)

**Figure 4.** (a) Fluorescence-labeled oligomeric M6P ligand; (b) confocal images of endolysosomal targeting. Reprinted with permission from [Hoogendoorn, S.; van Puijvelde, G. H. M.; Kuiper, J.; van der Marel, G. A.; Overkleeft, H. S. A Multivalent Ligand for the Mannose-6-Phosphate Receptor for Endolysosomal Targeting of an Activity-Based Probe. Angew. Chem. Int. Ed. 2014, 53, 10975–10978].

eties of this oligomeric M6P-ligands, a fluorescent-based BODIPY–DCG-04-oligomeric-M6P-cluster probe was used. This fluorescent-based probe showed its fluorescence activity in cysteine cathespins, an endo-lysosomal component, which plays a vital role in health and disease. In vitro cellular uptake and trafficking analysis of the BODIPY–DCG-04-oligomeric-M6P-cluster shows lysosome-targeting ability and it differs from its non-phosphorylated counterpart in both COS cells, a fibroblast-like cell line, and dendritic cells. However, the tedious synthetic steps associated with the solid-phase peptide synthesis approach, lack of polyvalency (polymeric), and the inclusion of several non-natural triazole moieties on the oligomeric peptide backbone limit their function as a natural protein mimic. Therefore, synthetic strategies for producing high-molecular-weight pendant M6P-ligands containing a glycopolypeptide polymer (Figure 4) as a mimic of M6P-moieties containing natural proteins were envisaged. Such glycopolypeptide polymers could help understand the natural protein complexity and lysosomal-targeting ability via the CI-M6PR-trafficking pathway.

**LYSOSOME-TARGETING M6P-LIGAND-BASED POLYPEPTIDE**

In 2016, Sen Gupta et al. demonstrated a synthetic route for synthesizing an end-functionalized pendant M6P-ligands containing a glycopolypeptide polymer (M6P-GP) (Figure 5A). The aim of synthesizing these M6P-GPs was selective delivery of therapeutic cargos inside the lysosomes via trafficking through the lysosomal pathway. The synthetic scheme involves incorporating the negatively charged M6P-ligands onto a poly-l-lysine backbone to maintain the required flexibility for the high-affinity binding ability to its corresponding M6PR.

![Figure 5](https://example.com/figure5.png)

**Figure 5.** (a) Chemical structure of end-functionalized M6P based glycopolypeptides; (b) lysosome targeting of the polyolipide on cell line; and (c) lysosome targeting with M6P-GP (FL-9b): MDA-MB-231 (a–d), L929 (e–h), and MCF-7 (i–l) cells were cultured for 4, 6, and 2 h, respectively, with FL-9b (200 μg/mL) in DMEM and then stained with LysoTracker Red (50 nM) for 30 min. The cells were probed by fluorescence microscopy. Merging of the FL signal (shown in green) and that of LysoTracker Red (shown in red) revealed colocalization as indicated by the yellow spots/areas (bars, 10 μm); the cyan color arrow indicates punctate-like vesicles. Cells were cultured for 4, 6, and 2 h, respectively, with FL-M6P-GPs (200 μg/mL) in DMEM and then stained with LysoTracker Red (50 nM) for 30 min. The cells were probed by fluorescence microscopy. Merging of the FL signal (shown in green) and that of LysoTracker Red (shown in red) revealed colocalization as indicated by the yellow spots/areas (bars, 10 μm); the cyan color arrow indicates punctate-like vesicles. Cells were cultured for 4, 6, and 2 h, respectively, with FL-M6P-GPs (200 μg/mL) in DMEM and then stained with LysoTracker Red (50 nM) for 30 min. The cells were probed by fluorescence microscopy.
NCA to ensure 100% glycosylation density on the side chains of the glycopolypeptide polymers.44

The in vitro cellular internalization studies of fluorescent-labeled M6PGPs in various cell lines (L929 mice fibroblast cells, MDA-MB-231 human breast adenocarcinoma cancer cells, and MCF-7 breast cancer cells) revealed that the M6P-ligand-containing glycopolypeptide polymers selectively trafficked into lysosomes using the cell surface CI-M6P receptor. This selective trafficking differs from non-phosphorylated pendant mannose-ligand-containing glycopolypeptide polymers that are more evenly distributed inside the cells. Additionally, maximum internalization of this M6PGP was observed in MCF-7 cells, which are known to overexpress M6PRs on the MCF-7 cell surface (Figure 5B,C).44

■ DELIVERY OF CARGO EXCLUSIVELY INSIDE LYSOSOMES USING M6P-POLYPEPTIDES

The delivery of cargo to lysosomes was attempted by encapsulating the cargo inside soft nanostructures such as micelles and polymersomes displaying the M6P-glycopeptide. The synthetic methodology developed by Sen Gupta et al. allowed the synthesis of amphiphilic M6PGPs to fabricate a self-assembled nanostructure with different morphologies for potential drug delivery application (Figure 6).45,49

The synthetic step involved the cycloaddition [copper-catalyzed azide−alkyne click reaction (CuAAC)] of alkynyl end-functionalized M6P-glycopeptide (Pr-M6P-AcGP15) and azide end-functionalized FDA-approved enzyme responsive polycaprolactone (N3-PCL) or acid-responsive polypropylene oxide (PPO)/polycaprolactone (PCL) as the hydrophobic block (Figure 6). The polypeptides’ amphiphilicity may be accomplished into various self-assembled nanostructures such as micelle, vesicle, nanorod, etc., depending upon the hydrophilicity/hydrophobicity ratio and the crystallinity nature of the hydrophobic segments.45

Enzyme- and pH-responsive soft micellar nanostructures that contained the M6P-glycopeptide on their surface could potentially encapsulate hydrophobic cargos were synthesized. These micellar nanostructures directly interact with the cell surface CI-M6P-receptor and internalize into the cells via CI-M6P-receptor-mediated endocytosis that subsequently traffics the cargo-encapsulated nanostructure into the lysosome. To better understand the trafficking pathway, cellular uptake experiments were performed with RBOE-loaded micellar nanostructures onto MDA-MB-231 adenocarcinoma breast cancer cells and MCF-7 breast cancer cells. The colocalization of red fluorescence from RBOE-containing micelle and the green fluorescence from LysoTracker green in epifluorescence microscopy imaging demonstrates the exclusive trafficking of RBOE-loaded M6PGP-based micellar nanostructures inside the lysosomes of both MDA-MB-231 cells and MCF-7 cells (Figure 7).45 Furthermore, the competition assays were conducted using the M6P monomer to investigate the involvement of CI-M6P-receptor during endocytosis onto MDA-MB-231 cells. The competition assay resulted in ~70% reduction of RBOE-loaded M6PGP-based nanocarriers inside the lysosome that highlights the predominant function of CI-M6P-receptors in the endocytosis pathway of the M6PGP-based nanocarriers.

![Figure 6.](https://doi.org/10.1021/acsomega.1c04771)
Finally, the inhibition assays were performed to show the possibility of clathrin-mediated endocytosis using several inhibitors such as chlorpromazine (clathrin inhibitor), genistin (caveolin inhibitor), and amiloride (macropinocytosis inhibitor). The use of chlorpromazine led to 32% inhibition of the uptake for the free polypeptides and their corresponding nanocarriers, indicating that the M6PGP- and M6PGP-based nanocarriers were endocytosed via the clathrin-mediated pathway.45

**TARGETED PROTEIN DEGRADATION USING M6P-BASED LYTAC AND GALNAC-BASED LYTAC**

Targeted protein degradation (TPD) technology has recently been of interest to researchers in academia and industry.50 Most therapies target individual proteins based on their specific activity, such as enzyme inhibition or ligand blocking. Recently, TPD platforms such as proteolysis-targeting chimeras (PROTACs) have been developed for proteins but are limited to those present in the cytosolic domains. In contrast, extracellular and membrane-associated proteins involved in diseases such as cancer or autoimmune disorders are difficult to address using TPD.50 However, one could degrade these extracellular proteins by using an “outside-in” strategy, bringing the targeted extracellular proteins inside the cell lysosomal compartment to access the cellular degradation machinery. For example, a cell surface protein receptor, low-density lipoprotein receptor (LDLR), binds to the proprotein convertase subtilisin kexin 9 (PCSK9) and promotes the trafficking of these binding complexes to the lysosome for degradation.50

Based on this concept, Bertozzi et al. have recently developed lysosome-targeting chimera (LYTAC) conjugates for degrading extracellular targets and cell membrane proteins.
associated with the lysosome targeting receptors (LTRs).\textsuperscript{46} They demonstrated this technology such that the endogenous LTRs can bind with extracellular glycoproteins and cell membrane proteins and exclusively shuttle them into the lysosomal compartment for degradation. The construction of LYTAC molecules consists of the conjugation of a ligand that is recognized by LTRs (that are recycled during the degradation process) and a target binding moiety such as small synthetic molecules or large molecules (i.e., antibodies and fragments), which recognizes the extracellular/cell membrane proteins. The developed LYTAC systems have capitalized on cation-independent M6P receptors (CI-M6PRs), whose principal function is to shuttle the lysosomal hydrolases to the lysosome, which is not cell-specific. However, the other LYTAC system has capitalized on Asialo-glycoprotein receptors (ASGPRs), whose function is the shuttling of components into the lysosome of the specific cells.\textsuperscript{47,48}

The synthetic LYTAC consists of non-hydrolyzable M6P glycopeptides as a booster for CI-M6PR (Figure 8A,C) with a target binder, which is nothing but an antibody to deplete soluble targets.\textsuperscript{46,47} These LYTACs can accelerate the degradation of several neurodegenerative disease-relevant targets, such as ApoE4. The broad distribution of CI-M6PR prompted the scientific community to develop the tissue-specific LYTAC by harnessing other LTRs.\textsuperscript{46,47} Another LTR, ASGPR, is exclusively expressed in the previously harnessed hepatocytes for antisense oligonucleotides and small interfering RNA delivery into the liver, which also trafficked the glycoproteins consisting of glycoproteins of N-acetylgalactosamine (GalNAc) or galactose into the lysosome.\textsuperscript{47,48}

The synthetic efforts of GalNAc-LYTACs included the conjugation tri-GalNAc ligand scaffold for receptor recognition and an azide-labeled antibody for target protein recognition. The cellular internalization by this GalNAc-LYTAC became dramatically more efficient than the M6Pn-LYTAC in the HEPG2 cells, primarily because of the higher expression of ASGPR compared to M6PR on these hepatocellular carcinomas cell lines (Figure 8). These GalNAc-LYTAC also mediate the cellular internalization of soluble cargos such as NeutrAvidin and IgG in HEPG2 cells. Studies regarding the LYTAC size suggested that the smaller LYTAC-cargo complexes are more efficient for the cellular internalized by ASGPR in HEPG2 cells (Figure 9).\textsuperscript{47,48}

Recently, an antibody-based PROTAC (AbTAC) has been developed as a complementary approach for the degradation of
cell membrane proteins. The LYTACs can access most of the membrane proteins that include excluded cytosolic domains, as the LYTAC can bind to the extracellular domain of the targets.\(^4\) In principle, the LYTACs can harness any LTR for the lysosomal degradation of targeted proteins. As the CI-M6PR is expressed in every cell surface, the M6Pn-LYTAC can bind and degrade cell surface membrane protein and extracellular target proteins in every tissue. However, the ASGPR is exclusively expressed in the liver cells, thus the LYTAC can specifically shuttle the extracellular and cell membrane target proteins in the liver cell lysosomes.\(^4\)

These developments for the lysosome-targeting degradation of extracellular and membrane proteins can potentially lead to therapeutic applications.

### FUTURE OUTLOOK

Recent synthesis of M\(^6\)GP\(_1\) has opened up the possibility of several biomedical applications. For example, M6P-based polymers can be used to target ligands to deliver therapeutics specifically inside the lysosome. In addition to their role in trafficking cargo specifically into the lysosome, MPRs are also known to overexpress during the early stage of several cancers, particularly in breast and prostate cancer. Therefore, M6P-labeled polymers can be displayed on the surface of nanocarriers to encapsulate and deliver drugs selectively to lysosomes of cancer cells that overexpress MPR. In this regard, M6P-based drug-loaded nanocarriers that can navigate the body, enter the tumor cells, traffic into the lysosome, and undergo fast disassembly to release the drug would be the most suitable platform. Delivery of lysosomotropic drugs via these M6P receptor-mediated therapeutic routes may induce the lysis of lysosomes and release the lysosomal proteins and enzymes into the cytosol, thus initiating LMP-mediated cell death. Such LMP-mediated cell death would lead to new approaches for targeted cancer therapy in breast and prostate cancer.

Conversely, in pancreatic ductal adenocarcinomas (PDA), pancreatic cancer cells are able to upregulate lysosomal biogenesis (to enhance nutrient recycling and stress resistance) due to the presence of the protein Myoferlin.\(^2\) Mechanistically, lysosomal localization of Myoferlin is necessary and sufficient for the maintenance of lysosome health and provides an early-acting protective system against membrane damage, thus allowing the cancer cells to proliferate. Myoferlin is upregulated in human pancreatic cancer and therefore designing inhibitors that block this protective function of Myoferlin may pave the way for future lysosome-centered strategies for the inhibition of PDA.

Additionally, end-functionalized M\(^6\)GP\(_1\) and related polymers allow easy attachment to the surface of various bioproducts, antibodies, and enzymes. Attachment of such polymers onto the surface of lysosomal hydrolases could help them deliver specifically into the lysosome of a diseased cell. Such an approach might be helpful for the treatment of LSD.

Finally, the discovery of PROTAC/LYTACs has opened a new horizon in TPD. Currently, the number of E3 ubiquitin ligase ligands used in the PROTAC technology is limited, restricting their subsequent application and development. Therefore, rather than overusing the same lysosome shuttling receptors, there is a need to exploit new lysosome-targeting receptors. For example, CD22 recycling receptors specifically expressing on B-cells or mannose receptors (MR, CD206) presenting on a tumor-associated macrophage surface may be good choices for cell-specific degradation. LYTAC provides possibilities to change protein inhibitor ligands into degraders. Inhibitors of proteins could be used as warheads during the design of LYTACs and thus can be used to deliver the proteins into the lysosome for degradation. This allows a possibility of several drugs that have not gained regulatory approval due to their side effects being repurposed as warheads of LYTACs. Additionally, drugs conjugated to LYTACs can be developed for drug-resistant cancer treatment in which the LYTACs target the proteins responsible for drug resistance, making the drug molecules effective. Finally, LYTAC-based nanoparticle delivery systems can also be developed to enable prolonged circulation and targeted delivery.

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### Notes

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