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Regulating Cellular Responses via Molecular Assembly in Cell Milieu for Cancer Therapy

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Abstract

Molecular assembly is a widely applied synthetic strategy for the construction of multimolecular architecture at a nanoscale or microscale that can simultaneously target numerous components. Upon the control over size, shape, valency, etc., molecular assembly can fulfill demands to regulate cellular responses even cell fate by approaching different cellular sub-organelles as potential cancer therapy.

Keywords: Molecular assembly | Cellular response | Cancer therapy

Introduction

Molecular assembly as a synthetic strategy can spontaneously form an array of well-defined nano/microstructures and has attracted much attention in various applications.[1] The assembly process is mainly governed by weak non-covalent bonds, such as ionic bonds (electrostatic interactions), hydrogen bonds, van der Waals interactions, and hydrophobic interactions. Although these forces are weak, their collective interactions can create stable structures. The design of synthetic molecular assembly often finds inspiration in Biology since they speak the same language of life. For this very same reason, molecular assembly can be designed to seamlessly integrate with the biological environment, and it is considered as the most promising approach to bionanotechnology.

Different from single molecules for which functional application solely relies on molecular components, molecular assembly offers a level of control over the characteristics of the molecular components and over the interactions among them leading to the most interesting applications at larger scale. At an

early stage of development of bionanotechnology, molecular assembly was mainly applied in pharmaceutical development of drug delivery systems. Various nano/microstructures have been developed for drug encapsulation to improve bioavailability and controllability.[2] Later, the expansion of imaging techniques advanced the understanding of the kinetics and surface characteristics of molecular assembly. Scientists found out that the dynamic process of synthetic molecular assembly could stimulate cellular responses via interface communication. Therefore, constructing molecular assembly in the cell milieu is an emerging bionanotechnology leading pharmaceutical development into a new era.

The goal of this work is to highlight the most recent studies on cellular sub-organelle-targeted molecular assembly comprising design of targeted molecular assembly, exclusive features of subcellular targeted molecular assembly, and the applications in biomedicine for cancer treatment.

Plasma Membrane-targeting Molecular Assembly

The plasma membrane has long been an attractive target in the development of therapeutics regarding its multitude functions.[3] The bilayer structure separates and protects the interior of the cell from the outside environment, serves as the attachment surface for extracellular matrix, and mediates signaling processes from outside into the cell interior. Targeting plasma membrane to enhance its disruption has been established as the fundamental strategy for the development of antibiotic drugs.[4] Until the past decade, targeting membrane proteins regulating signaling pathways involved in the formation and progression of human cancers emerged in cancer treatment. However, the lack of ubiquitously expressed tumor-specific antigens or receptors on cancer cells and the geno/phenotypic heterogeneity of tumors limited the application of such methods. During the past few years, scientists have developed plasma membrane-targeted molecular assemblies reaching cancer cell selectivity.

In 2014, the Xu lab reported the first plasma membrane-targeted molecular assembly that selectively targeted cancer cells. In that research, cancer biomarkers, surface and secretory alkaline phosphatases (ALPs) dephosphorylate a small D-peptide derivative **PM1** in pericellular space triggering molecular assembly into nanonets around cancer cells that overexpress phosphatase (Figure 1).[5] By blocking cellular mass exchange between the cancer cells with their environment, the nanonets significantly decrease the cancer cell viability. Carbonic anhydrase (CA) IX overexpressed on cell membranes of hypoxic tumors was also applied in enzyme-assisted plasma-membrane targeted molecular assembly. The Chen lab coupled CA IX inhibitor 4-(2-aminoethyl)benzenesulfonamide (ABS) with D-peptide derivatives obtaining molecule **PM2** that self-assemble on hypoxic cancer cell surfaces causing intracellular acid vesicle damage and blocking protective autophagy (Figure 1).[6]

Inspired by the Xu lab's pioneering concept of using cellular enzymes to trigger the release of activated compounds that can form molecular assembly structures *in situ* in the vicinity of the initiating enzymes, our lab designed and synthesized a ruthenium-complex-based D-peptidic molecule **PM3**. Instead of targeting the whole membrane, the Ru-complex **PM3** selectively initiates molecular assembly into nanofibrils on lipid rafts

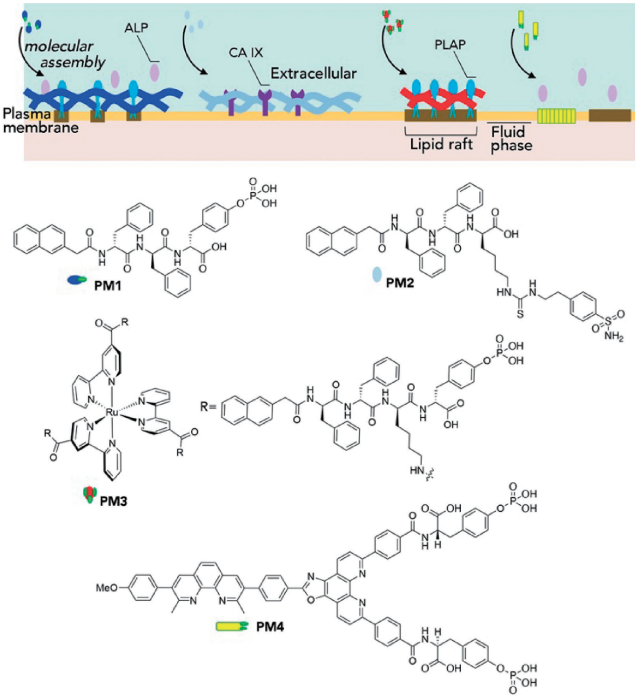

Figure 1. Schematic presentation of enzyme-assisted plasma membrane-targeting molecular assembly and the chemical structures of precursor molecules.

(Figure 1),[7] which substantially increases the sub-cellular resolution of molecular assembly technologies. The lipid-raft-targeted molecular assembly suppresses cervical cancer cell migration and invasion via actin cytoskeleton mediated biophysical regulations leading to potentially precise control of cell response. Our lab later synthesized an expanded π-conjugation-based molecular precursor **PM4**, which has an extremely high rigidity and a long hydrophobic length that is comparable to the hydrophobic width of plasma membrane.[8] Guided by the ALPs in cancer environment, the soluble precursors transform into hydrophobic monomers forming assemblies inserted into the fluid phase of the plasma membrane exclusively (Figure 1), which destroys the selective membrane permeability gradually resulting into cancer cell death.

In 2020, our lab reported integrin-ligand binding induced molecular assembly on cancer cell membrane (Figure 2). A self-assembling integrin ligand **PM5** was synthesized by coupling integrin β1 ligand YIGSR with several aromatic amino acids.[9] Upon cell culture, these molecules selectively form nanofibrous microdomains via molecular assembly on the apical membrane of glioma cells inhibiting their migration and invasion without introducing cytotoxicity. We also reported a molecular design for integrin and heparan sulfate dual-targeting molecular assembly on cancer cell membrane. In this research, we decorated IKLLI, a ligand of both integrin β1 and heparan sulfate, with aromatic amino acids to obtain self-assembling ligand **PM6**. Molecule **PM6** selectively targets cancer cell membrane by forming nanofibrils on the plasma membrane (Figure 2). Without inducing toxicity, the nanostructures suppress cancer metastasis. These studies demonstrate a new membrane-targeted molecular assem-

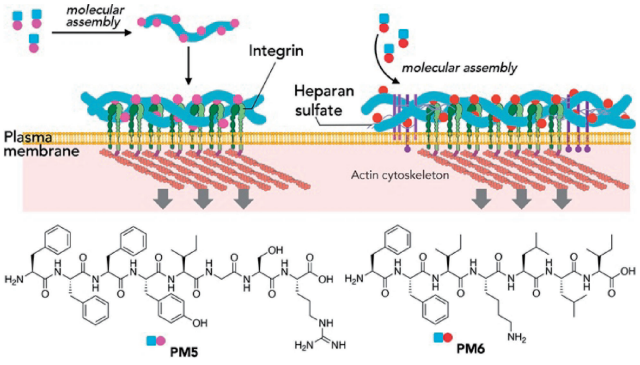

Figure 2. Schematic presentation of integrin-targeted molecular assembly and integrin/heparan sulfate dual-targeting molecular assembly and the chemical structures of self-assembling ligands.

bly via protein-ligand binding interaction which is different from enzyme-assisted self-assembly strategy.

ER-targeted Molecular Assembly

Endoplasmic reticulum (ER) is an elaborate network membrane that extends throughout the cell. The ER is closely associated with plasma membrane, mitochondria, Golgi, vacuoles, peroxisomes, late endosomes and lysosomes for coordination activities. The functions of ER include protein synthesis that occurs in the rough ER, lipid metabolism that occurs in the smooth ER, and detoxification of the cell. The capacity of the ER to process proteins is limited. The accumulation of unfolded and misfolded proteins can lead to ER stress initiating apoptosis which has been encountered as an anticancer strategy.[10] Inhibiting components of the unfolded protein response with small molecule inhibitors is one of the most common approaches of drug discovery as anti-tumor agents.

The Xu lab reported an ER-targeted design of molecular assembly using phosphotetrapeptide **ER1**. Attaching an L-amino acid at the C-terminal of a D-tripeptide result in crescent-shaped molecular assemblies via ALP triggered dephosphorylation.[11] The crescent-shaped nanostructures disrupt plasma membrane integrity to enable further accumulation on the ER causing ER stress and activating the caspase signaling cascade for cell death (Figure 3). Based on this design concept, the Xu lab integrated the ER-targeting peptide building block with naproxen–a non-steroidal anti-inflammatory drug (NSAID) and ligand of cyclooxygenase-2 (COX-2). The phosphatase triggered molecular assemblies on ER enable the sequestration of COX-2 and protein-tyrosine phosphatase 1B (PTP1B) on ER demonstrating a new strategy for modulating protein-protein interactions.[12]

Inspired by the research on micelles, liposomes, and polymeric particles that targeted the ER, the Basu lab designed and synthesized triazine-based small molecule **ER2** containing a fluorescent dansyl group as the ER targeting moiety that formed a self-assembled hexameric rosette structure through Watson-Crick based H-bonding with 5-fluorouracil leading to the formation of hitherto spherical nanoparticles. These nanoparticles internalized into HeLa cervical cancer cells by micropinocytosis and specifically localized into the ER to induce ER stress (Figure 3).[13]

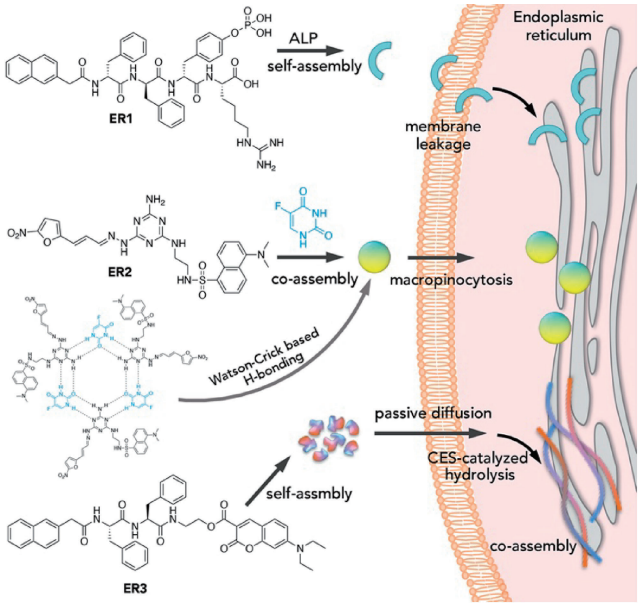

Figure 3. Schematic presentation of three different designs of ER-targeting molecular assembly and the chemical structures of precursor molecules.

Our lab recently reported a carboxylesterase (CES)-facilitated ER-targeting molecular assembly.[14] We designed and synthesized an *N*-hydroxylethyl peptide by coupling naphthalene-Phe-Phe with ethanolamine. By protecting the hydroxyl group using a fluorescent coumarin derivative via an ester linkage, a precursor molecule **ER3** was obtained forming sparse aggregates via molecular assembly in water. After passive diffusion, aggregates entered cytoplasm and were hydrolyzed into *N*-hydroxylethyl peptide and coumarin derivative by intracellular carboxylesterase (CES). The hydrolyzed product molecules co-assembled into nanofibrils around the ER inducing ER dilation (Figure 3). In this research, the comparison of **ER3** with the other two self-assembling molecules also indicated that vesicle-shaped assemblies or sparse irregular-shaped aggregates facilitate passive diffusion into cytosol approaching the ER.

Mitochondria-targeting Molecular Assembly

Besides oxidative phosphorylation, central carbon metabolism, and the biosynthesis of intermediates for cell growth, the most notably key roles that mitochondria play in the cell, mitochondria participate in nearly all aspects of cell function and fate.[15] They are the powerhouse of cell, and the suicidal weapon store inside the cell. Therefore, inhibiting tumor-specific alterations of the mitochondrial metabolism or stimulating mitochondrial membrane permeabilization are considered as promising therapeutic approaches.[16] The development of small molecule drugs mainly focused on targeting permeability transition pore complex (PTPC) in the mitochondrial outer membrane or inducing the overproduction of reactive oxygen species (ROS). Because directly targeting mitochondria has the potential to bypass resistance mechanisms, increasing the level of selectively of mitochondrially-targeted anticancer agents is a promising avenue for further research.

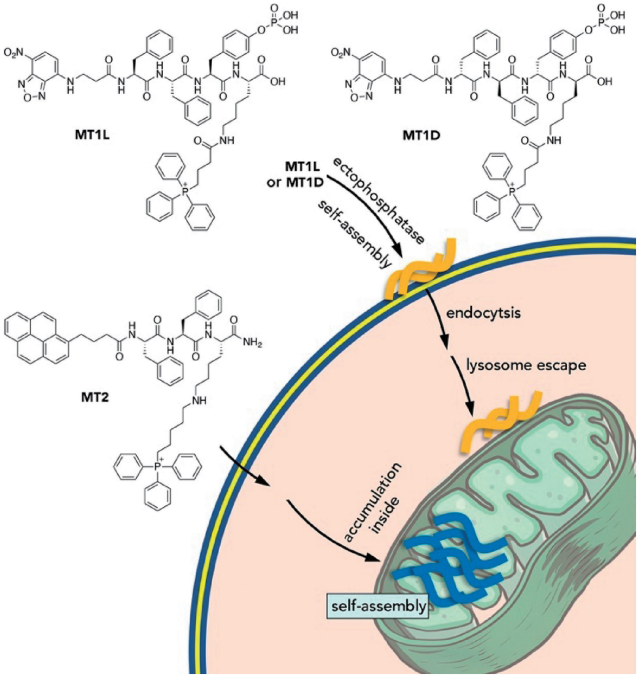

Figure 4. Schematic presentation of TPP-based mitochondria-targeting molecular assembly and the chemical structures of candidate molecules.

In 2016, the Xu lab reported a bioinspired molecular process that selectively generated the assemblies of redox modulators selectively targeting the mitochondria of cancer cells.[17] By attaching triphenyl phosphonium (TPP)–a facile molecular motif for targeting the mitochondrial matrix,[18] to a pair of enantiomeric, phosphorylated tetrapeptide derivatives, a pair of precursors **MT1L** and **MT1D** were synthesized. Upon dephosphorylation catalyzed by ectophosphatases overexpressed on cancer cells, the precursor molecules self-assemble to form nanoscale assemblies only on the cancer cell surface. Following a caveola/raft-dependent endocytosis, these nanoscale assemblies enter into the cancer cell, escape from the lysosome, induce dysfunction of mitochondria and result in cell death (Figure 4). Later, the Ryu lab synthesized **MT2** which consisted of diphenylalanine as a β-sheeting-forming building block, TPP, and pyrene as a fluorescent probe. **MT2** favorably accumulated in the mitochondria of cancer cells leading to self-assembly forming nanofibrils (Figure 4). The fibrils destroyed the mitochondrial membrane and activated the intrinsic apoptotic pathway against cancer cells.[19]

Besides using TPP, the Xu lab found that conjugating a hydrophilic FLAG motif (DDDDK) to self-assembling motifs afforded precursors **MT3** assembling into micelles.[20] After being taken up by cells, upon the enterokinase (ENTK) catalyzed cleavage of the FLAG, the micelles turn into nanofibers to locate mainly at mitochondria. Besides being applicable as drug carriers, further engineering of the MitoFlag in **MT4** facilitated the trafficking of a histone protein (H2B) to the mitochondria in cancer cells selectively,[21] which is potentially applicable in manipulating inter-organelle communications (Figure 5). Replacing the self-assembling motifs with a lipid-like moiety (NH_2-Glu-$(C16)_2$), **MT5** was obtained maintaining

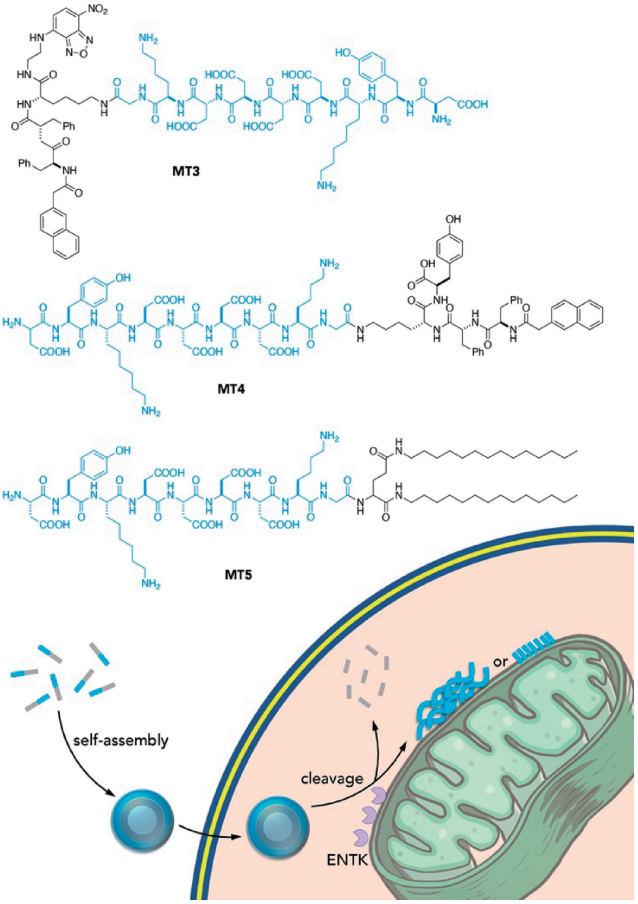

Figure 5. Schematic presentation of MitoFlag-based mitochondria-targeting molecular assembly and the chemical structures of candidate molecules.

the same self-assembly performance forming micelles for mitochondria-targeted drug delivery.[22]

Dual-targeting Molecular Assembly

Combination treatments are the current trends leading anticancer drug research and development. Compared to single drugs, drug combinations that target multiple biological pathways take advantage of synergistic killing and decrease the risk of relapse. Regarding the R&D cost, the greatest efforts on the development of combination therapy are concentrated on the screening of small molecule drug combinations. The promise of nanomedicine in the fight against cancer motivates the enthusiasm to elevate the development of nanotechnology for combination therapy. And the combination of molecular assembly in cell milieu is proved to be a promising approach.

Our lab developed a strategy of producing small self-assembling molecules for dual-targeting combination treatment. As demonstrated in Figure 6, chlorambucil, a small molecule chemotherapy medication that alkylates and crosslinks DNA was coupled with ER-targeting self-assembling motifs (L and D-version) via diester bonds to obtain **DT1L** and **DT1D**. Both molecules self-assemble into nanovesicles that easily enter into the cells.[23] Upon the hydrolysis by CES that is commonly

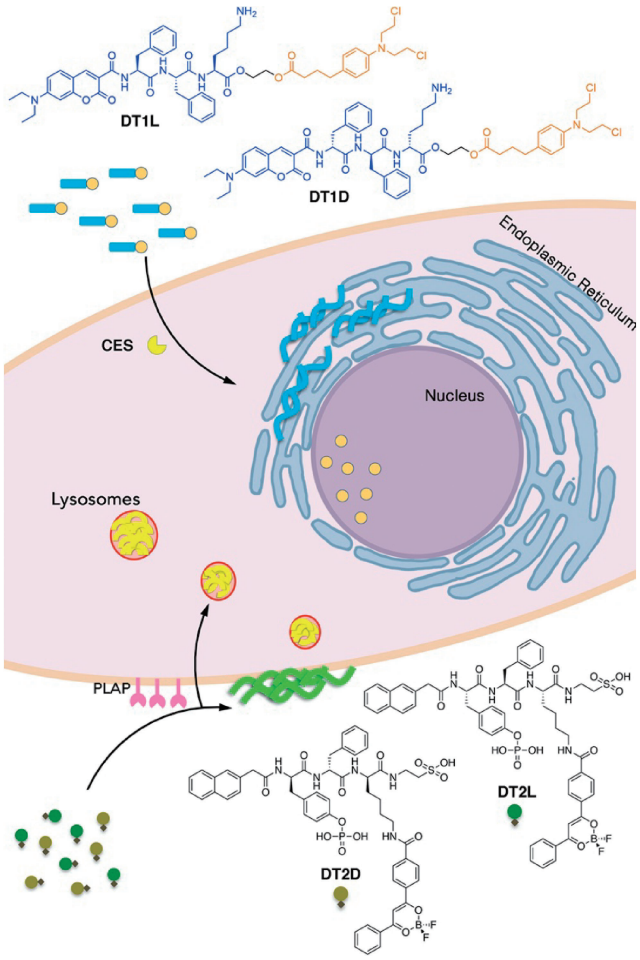

Figure 6. Schematic presentation of two dual-targeting molecular assembly design strategies for combination treatment of cancer, and the chemical structures of candidate molecules.

expressed in tumor tissue, chlorambucil is released to damage DNA, and the self-assembling motifs are released to form nanofibrils on ER inducing ER stress (Figure 6). The dual-targeting design reached higher anticancer efficacy than single-targeting drug.

We also demonstrated another strategy of dual-targeting molecular assembly design by co-administration of a pair of homochiral-peptide derivatives targeting two different suborganelles for molecular assembly. Naphthalene-ᴅTyr-Phe-Lys-taurine and naphthalene-ᴅtyr-phe-lys-taurine were selected to couple with the aggregation-induced emission (AIE) motif–boron diketone on the side chain of lysine obtaining **DT2L** and **DT2D**. Mediated by the PLAP enzyme on cancer cell membrane, plasma membrane and lysosome dual-targeted molecular assemblies introduces a synergistic anti-cancer effect with amplified efficacy (Figure 6).[24] Such research opens up new opportunities for the design of combination therapy via the application of nanotechnologies.

Conclusion

Instead of acting solely as pharmaceutical drug carriers, scientists have discovered more functions of molecular assemblies by constructing nano-biointerfaces to facilitate interface communications between synthetic nano/microstructures and cellular organelles. Considering the requirement of noninvasive *in-situ* self-assembly tracking *in vitro*/*in vivo*, small organic fluorophore, for example 7-nitrobenzofurazan (NBD), coumarin derivatives, pyrene, BODIPY, etc.; transition metal complex, for example ruthenium (II) and iridium (III) with chelating N-heterocyclic ligands; and expanded π-conjugation-based molecules have been used in molecular design to make self-assembly illuminated in biological systems. Besides considering the excitation/emission properties of the fluorophores for biocompatibility, as a hydrophobic building block or three-dimensional scaffold, the fluorophores are also applied to regulate the overall hydrophilicity/hydrophobicity, charge density of the target molecules facilitating the formation self-assembled morphologies with different surface 'function'. Various possibilities are generated via molecular assembly instructed interface interactions including the control of membrane permeability, the regulation of membrane associated proteins, and the adaptive remodeling of the membrane structures. As summarized here, most developments were made on targeting sub-cellular organelles with large membrane structures that are easily targeted by nano/microstructures exhibiting powerful anti-cancer effect. The design of molecular assembly targeting small compartments with higher resolution on plasma membrane and intracellular organelles of smaller size, like endo/lysosomes and cytoskeleton filaments, is still underdeveloped. Advancing the controllability of molecular assembly in both morphology, selectivity, and functionality via bottom-up approaches is desired in future study.

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