



Review article

From oncolytic peptides to oncolytic polymers: A new paradigm for oncotherapy

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ABSTRACT

Traditional cancer therapy methods, especially those directed against specific intracellular targets or signaling pathways, are not powerful enough to overcome tumor heterogeneity and therapeutic resistance. Oncolytic peptides that can induce membrane lysis-mediated cancer cell death and subsequent anticancer immune responses, has provided a new paradigm for cancer therapy. However, the clinical application of oncolytic peptides is always limited by some factors such as unsatisfactory bio-distribution, poor stability, and off-target toxicity. To overcome these limitations, oncolytic polymers stand out as prospective therapeutic materials owing to their high stability, chemical versatility, and scalable production capacity, which has the potential to drive a revolution in cancer treatment. This review provides an overview of the mechanism and structure-activity relationship of oncolytic peptides. Then the oncolytic peptides-mediated combination therapy and the nano-delivery strategies for oncolytic peptides are summarized. Emphatically, the current research progress of oncolytic polymers has been highlighted. Lastly, the challenges and prospects in the development of oncolytic polymers are discussed.

1. Introduction

Cancer is a major and increasing public health problem worldwide, which leads to high morbidity, mortality, and economic burden. In 2020, there were an estimated 19.3 million new cases and 10.0 million deaths from cancer worldwide, and the annual number of new cancer cases would increase to 28.4 million by 2040 if the incidence rates remain unchanged [1]. At present, first-line cancer treatments including chemotherapy, radiotherapy, and immunotherapy have improved outcomes for cancer patients to some extent [2]. However, these treatments still have some limitations, for instance, chemotherapy and radiotherapy usually lead to therapeutic resistance as well as severe side effects, while immunotherapy only has low response rates in clinics [3–5]. Moreover,

due to the genetic/epigenetic diversity of cancer cells and high selective pressure imposed by the tumor microenvironment (TME), extensive tumor heterogeneity was found in different patients, different tumor sites, and even within a single tumor [6–8]. For example, vascular abnormalities and high interstitial fluid pressure limit the penetration of traditional drugs to the tumor center, and the cancer cells in the hypoxic regions are thought to remain dormant and probably facilitate tumor invasion [9,10]. As a result, treatment-effect heterogeneity would be caused by tumor heterogeneity, and the therapy-resistant residual cancer cells will eventually lead to tumor recurrence and metastasis, which are the main reasons for treatment failure and death associated with cancer [11]. Therefore, the development of a new general strategy has become urgent to overcome the clonal heterogeneity or resistance.

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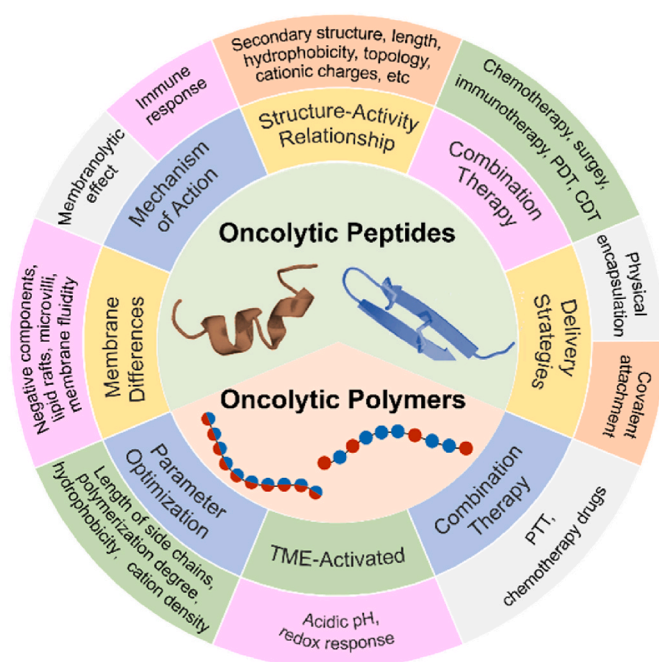
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Over the past two decades, a novel class of anti-tumor agents, named oncolytic peptides, has attracted much attention and interest in both basic research and clinical application (Scheme 1). Oncolytic peptides are derived from, or inspired by, natural host defense peptides (HDPs)/antimicrobial peptides (AMPs) that are produced in a wide variety of life forms. These peptides exhibit plasma membrane-lytic activity specifically targeting malignant cells [12,13]. Oncolytic peptides typically are composed of positively charged amino acids and hydrophobic amino acids, with a cationic amphiphilic structure and high penetration [14]. Due to the plenty of negative charges on the tumor cells' surface, oncolytic peptides would be adsorbed onto the membrane surface via electrostatic interaction firstly, eventually resulting in perturbation of the membrane and tumor cells lysis after the membrane-bound peptides reached a threshold concentration [15,16]. Unlike conventional cytotoxic agents, targeted agents, hormone-based drugs, and antibody drugs that act within various signaling pathways or interact with specific intracellular targets, the targets of oncolytic peptides are the negatively charged membrane components, which are uniquely but homogeneously present on almost all cancer cells [13]. Because it would be probably a “costly” solution for most cancer cells to reprogram the organization and/or composition of the membrane lipids, the mode of action of oncolytic peptides not only appears to bypass the tumor innate therapy resistance but also reduce the risk of acquired resistance [17,18]. And this relatively non-specific mode of action endows oncolytic peptides with the ability to kill tumor cells within different TME and even the “dormant” tumor cells or cancer stem cells, which makes them promising agents for destroying the tumors with high heterogeneity [13,19,20]. This strategy should be superior to the traditional photodynamic therapy (PDT), photothermal therapy (PTT), and chemodynamic therapy (CDT), whose antitumor effects are highly limited by the penetration depth of light or reductive TME [21–25]. In addition, besides acting directly on cancer cells and causing oncolysis, some oncolytic peptides can also induce the exposure of tumor-associated antigen (TAA) and release of danger-associated molecular pattern molecules (DAMPs) from dying tumor cells, thereby triggering systemic anticancer immune responses [26].

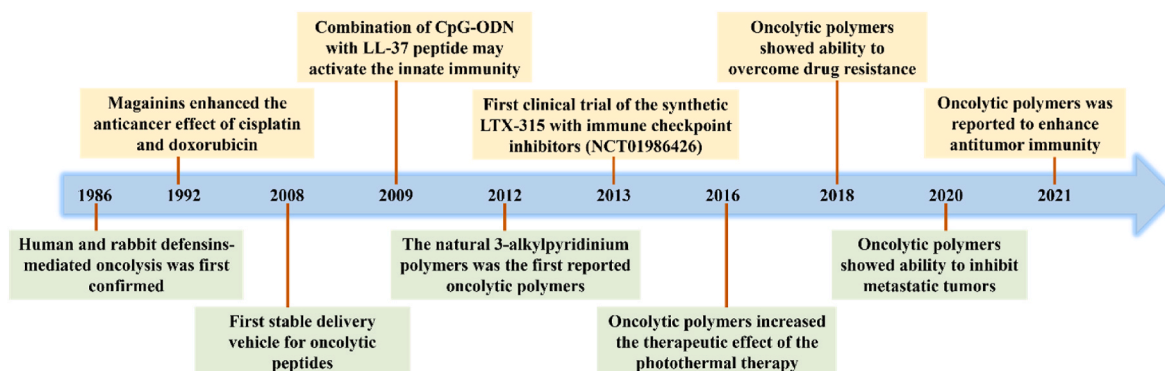
Although oncolytic peptides have broad prospects in combating malignant tumors, clinical application of the oncolytic peptides is still hindered by the unsatisfactory bio-distribution, poor bioactivity & stability *in vivo*, toxicity to normal cells, and high manufacturing costs [27–29]. Thanks to the recent rapid development of nanotechnology, various nano-delivery systems have been designed for improving the anticancer efficacy of oncolytic peptides *in vivo*. In a nutshell, these strategies can prevent the premature release of oncolytic peptides during blood circulation, realize the physiology/external stimuli-controlled release of oncolytic peptides within solid tumors, protect oncolytic peptides from proteolytic attack and reduce the systemic toxicity, etc [30,31]. However, these nano-delivery systems do not address the issue of high-cost peptide-based drugs, and fabrication of the sophisticated

oncolytic peptides nano-assemblies may in turn increase the synthetic complexity and total cost [31]. As a result, recently we and other labs have focused on developing oncolytic peptides-mimicking synthetic amphiphilic cationic polymers (defined as **oncolytic polymers**) for tumor treatment [32,33]. Due to the good chemical modifiability, high stability, low cost, and ease of mass production and storage, oncolytic polymers are emerging as a potential alternative to oncolytic peptides, which both represent a promising new therapeutic paradigm to complement traditional cancer treatment.

The advances in HDPs/AMPs with anticancer activity have been summarized in several review papers, which either focus on the tumor-suppressor mechanisms (membranolytic or non-membranolytic mode), factors affecting the anticancer activities, or the application in different tumor types [15,18,19,29,34–40]. Furthermore, Yang and Pan et al. recently summarized the application and prospects of AMPs-mimicking synthetic macromolecules and other peptidomimetics in combating cancer/microbial infection, respectively [32,41]. However, oncolytic peptides, as a major member of the HDPs/AMPs family possessing membrane lytic activity, and the emerging oncolytic polymers in the past few years also require special attention and systematic understanding. In this review, we provide overviews of the oncolytic peptides



Scheme 2. Schematic overview of the development of oncolytic peptides & oncolytic polymers and potential opportunities for oncolytic polymers.



Scheme 1. Timeline illustrating the history of the development from oncolytic peptides to oncolytic polymers.

and oncolytic polymers-based therapeutics for cancer therapy (Scheme 2). Specifically, the function mechanisms of oncolytic peptides and strategies for optimizing the biological activity of oncolytic peptides were first summed up. Then we will review the application of oncolytic peptides in combination with cancer therapy, emphasize the advances in stimuli-responsive oncolytic peptides and delivery strategies for oncolytic peptides, highlight the latest research development of oncolytic polymers, and discuss the challenges as well as future outlook in oncolytic polymers-mediated cancer therapies.

2. Function mechanism and structure-activity relationship of oncolytic peptides

2.1. Membrane differences between normal cells and cancer cells

Based on the differences in components and structure of plasma membranes between normal cells and cancer cells, oncolytic peptides may achieve some degree of selectivity against tumor cells. First, it was reported that cancer cells possess more negative charges on the outer surface of the plasma membrane, which lead to a stronger electrostatic attraction between cationic oncolytic peptides and tumor cells [42]. The increased negative charges on tumor cells come from the following reasons: (1) anionic phosphatidylserine is mainly located in the inner leaflet of the membrane of normal cells, while the outer leaflet contains neutral lipids including sphingomyelin and phosphatidylcholine. However, cancer cells lose this membrane asymmetry due to the stress conditions (e.g., acidity, hypoxia, and excess reactive oxygen species (ROS)) in the TME, resulting in plenty of phosphatidylserine exposed on the outer leaflet of the membrane [43,44]; (2) sialic acid, an anionic motif linked to *O*-glycosylated mucins and gangliosides, is overexpressed on external surfaces of many cancer cell lines [45–47]; (3) abundant proteoglycans are located on external surfaces of tumor cells, which contain high negatively charged glycosaminoglycan motif such as heparan sulfate and chondroitin sulfate [48,49]; (4) hyaluronan, an anionic glycosaminoglycan adsorbed to membrane protein via non-covalently interaction, is also overexpressed within tumor tissue [50] (Fig. 1). Although most studies show that the increased negative charges on cancer cells' membrane lay the foundation for the oncolytic peptides-mediated oncolysis, Fadnes et al. reported that high expression of heparan sulfate on the outer membrane surface would keep the LfcinB and KW5 peptide away from the lipid bilayer and finally inhibit their oncolytic activity [51]. Therefore, more detailed research is needed to further clarify the exact role of each negatively charged component on tumor cells membrane.

Apart from the difference in charge properties of cells membrane, it was reported that cancer cells have more microvilli coated on the surface than normal cells, and this morphological structure provided tumor cells with a larger oncolytic peptides-accessible surface area [52,53]. Since oncolytic peptides-mediated membrane disruption is a concentration-dependent process, the increased membrane surface area favors the adsorption of oncolytic peptides and makes it easier to reach the threshold peptide concentration on the membrane surface [16,54,55].

The third reason for the selectivity of oncolytic peptides is the increased fluidity of the plasma membrane in many malignant cell lines such as lung cancer, lymphomas, and gliomas [42,56,57]. This could be because membrane fluidity and stiffness are mainly regulated by the membrane cholesterol content, and both of these cancer cells display the characteristic of decreased cholesterol levels. Conversely, a high level of sterol is present in the membrane of normal eukaryotic cells such as erythrocytes, which prevents the membrane-adsorbed oncolytic peptides from inserting into the interior of the lipid bilayer and thereby increases the tolerance of normal cells [58]. However, lipid rafts, the detergent-resistant region in the plasma membrane composed of cholesterol and sphingolipids, are recently found to significantly increase in the prostate and breast cancer cells [59]. The abundant membrane lipid rafts can increase the mechanical durability of the membrane structure, thereby making these cell lines less susceptible to oncolytic peptides-mediated membrane damage [60–62].

Therefore, the differences in outer membrane composition and structure have been implicated to make cancer cells more sensitive to membranolysis than normal cells. Though there are some exceptions for the interaction between different cancer cell lines with certain oncolytic peptides, the membrane-related factors are suggested to influence the oncolytic peptides-membrane interaction in a general manner [19]. Briefly, the abundant anionic components and microvilli on the membrane can drive the attraction and accumulation of cationic oncolytic peptides on the cell surface, while the increased membrane fluidity owing to reduced cholesterol content eventually facilitates insertion of oncolytic peptides into the lipid bilayer. Additionally, some oncolytic peptides can specifically bind to the overexpressed membrane proteins on cancer cells' surface, for example, the NK-2 peptide derived from NK-lysin was reported to co-localize with *P*-glycoprotein on the multi-drug resistance (MDR) cancer cells, thereby efficiently eradicating the *P*-glycoprotein-positive MDR cells within the heterogeneous tumor [63].

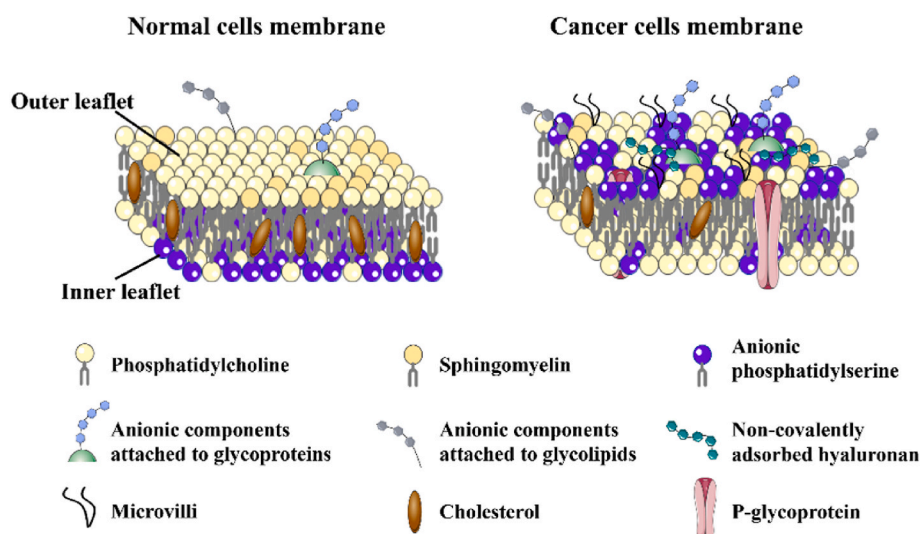


Fig. 1. Schematic diagram illustrating the difference between normal cells membrane and cancer cells membrane. Briefly, the exposed negatively charged phosphatidylserine on membrane outer leaflet, the increased expression levels of anionic components (e.g., sialic acid and glycosaminoglycan attached/adsorbed to glycoproteins and/or glycolipids) on membrane outer leaflet, the presence of microvilli on the membrane, the decreased cholesterol level and increased expression level of *P*-glycoprotein contribute to the selectivity of oncolytic peptides (oncolytic polymers) towards tumor cells.

2.2. Membranolytic mode of actions of oncolytic peptides

Compared to normal eukaryotic cells, most of bacteria and cancer cells have similar membrane structures and properties, which also explains why oncolytic peptides can induce cancer membrane lysis in a similar mechanism to the AMPs-mediated disruption of bacterial membrane [18]. Although the underlying interaction mechanism between oncolytic peptides and plasma membrane is not fully elucidated, several techniques have been employed to characterize the mode of action of oncolytic peptides towards tumor cells, such as X-ray diffraction, surface plasmon resonance, attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectroscopy, confocal fluorescence microscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), circular dichroism (CD) spectroscopy, flow cytometry and molecular dynamics simulation (MD simulation), etc. [64–70]. In general, the oncolytic peptides-mediated cancer cell killing can be roughly divided into four main stages: (1) oncolytic peptides are attracted to the cancer cell surface via electrostatic interaction between the negatively charged cytoplasmic membrane components and the positively charged peptides residues [65]; (2) additional hydrophobic interactions between the phospholipid hydrophobic tail and the residues of the non-polar peptide serve to facilitate the embedding of oncolytic peptides into tumor cells membranes via multiple theoretical models [38]; (3) the membrane curvature would be changed, subsequently, the structure and integrity of the cells membrane were disrupted (e.g., blebbing, pore formation, and vascularization) [71]; (4) tumor cells are lysed and the intracellular contents would be released [68]. It is worth mentioning that most of oncolytic peptides are unfolded and inactive in an aqueous solution, whereas it would preferentially fold at the surface of tumor cells, thereby adopting a specifically amphiphilic secondary structure (e.g., helical conformation or β -structure) capable of membrane lysis [69]. For instance, Schneider et al. have designed an 18-residue SVS-1 oncolytic peptide, which is unstructured in solution or the presence of neutral lipid vesicles [67]. However, when SVS-1 was incubated with tumor cells membrane-mimicking negatively charged lipid vesicles, the resulting CD spectrum suggested that SVS-1 was driven by electrostatic interaction to adopt a β -sheet structure consistent with hairpin formation. Since SVS-2 (a control peptide that adopts a mixture of β -sheet structure and random coil, but is unable to fold into a hairpin conformation) was inefficient to induce leakage of negatively charged liposomes or kill any of the tumor cell lines, the β -hairpin conformation folding process is requisite for realizing the membranolytic function of the SVS-1 peptide.

Among the above-mentioned theoretical modes of action between oncolytic peptides and cancer cells membrane, the most common models are the carpet-like model, barrel-stave model, and toroidal pore

model (Fig. 2). In the carpet-like model, cationic oncolytic peptides (e.g., ovispirin, cecropins, dermaseptin, and LL-37 peptides) are usually performed as highly helical structures, which bind to the negatively charged membrane surface via interacting mainly with the headgroup of the phospholipids. According to the results of linear dichroism (LD) spectroscopy and MD simulation, the helical parts of these oncolytic peptides basically align parallel to the surface of tumor cells in a carpet-like fashion [69,72–75]. After the critical peptides concentration is reached, the phospholipids order and packing would be disrupted, which could be confirmed by the perturbations of the phosphate & choline groups of the lipids and the lipid ester & acyl chain vibrations observed by ATR-FTIR spectroscopy. As a result, the cell membrane stability and permeability would be disturbed, thereby leading to membrane disintegration in a detergent-like manner and tumor cell lysis. In the barrel-stave model, oncolytic peptides are aggregated and inserted perpendicularly into the membrane, meanwhile, the hydrophobic region of the amphiphilic peptides tends to bind to hydrophobic phospholipid tails [76,77]. As a result, a peptide bundle would be formed in the membrane with a central aqueous lumen, much like a barrel composed of helical peptides as the staves. And the pore size would become larger with the increased number of aggregated oncolytic peptides, finally resulting in cytoplasmic contents leakage and cell death [61,78]. This mechanism requires that the peptides possess sufficient backbone length to span the cell's membrane lipid bilayer, therefore this model usually applies to the peptides containing more than twenty amino acid residues (e.g., alamethicin) [61,70,79]. According to the data of oriented circular dichroism, neutron scattering, and synchrotron-based X-ray scattering, the alamethicin-induced transmembrane pores consist of 3–11 parallel helical alamethicin molecules, which would be modulated by changing the composition of bilayer lipids. In addition, the approximate thickness of the transmembrane channel is 1.1 nm, which is consistent with the diameter of the alamethicin helix [76]. Differing from the barrel-stave model, in the toroidal pore model, oncolytic peptides always bind to the negative phospholipid head groups throughout the process of inserting these peptides into the lipid bilayer. Due to the continuously increased degree of membrane bending, the aqueous toroidal pore is formed, which is composed of the membrane lipid head groups and the embedded peptides [41,80]. In this process, the positively polar faces of the oncolytic peptides associate with the negatively polar head groups of the phospholipids. The lipids then tilt and connect the two leaflets of the membrane, creating a continuous bend in the shape of a toroidal pore from top to bottom. Because the pore is lined by both the cationic peptides and the anionic lipid head groups, the peptide charges could be screened and masked, thereby leading to a reduction in Coulomb energy and maintaining the toroidal pore stability [76]. Finally, cancer cells could be killed because

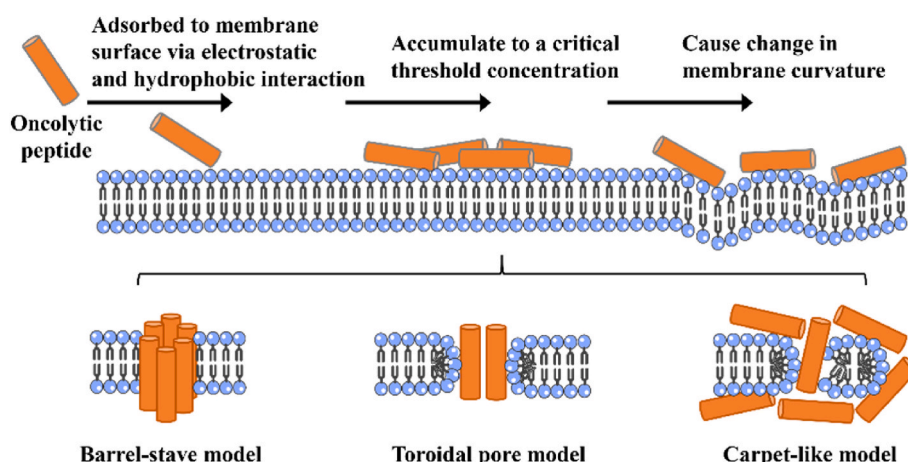


Fig. 2. Schematic diagram illustrating the membranolytic mechanism of actions of oncolytic peptides and some representative acting modes of oncolytic peptides.

of the loss of membrane integrity, depolarization of the plasma membrane, and leakage of intracellular contents. In addition, the generated toroidal pore was unstable due to the increased membrane tension, which may lead to membrane perturbation and pore disintegration. As a result, some peptides (e.g., magainins 2, protegrin-1) may be released into the cells and further bind to the intracellular targets, such as DNA and RNA, thereby inhibiting the essential pathways such as DNA replication and protein synthesis [77,81].

In addition to the well-accepted three models, there are still some models suitable for describing the interaction between certain oncolytic peptides and eukaryotic cells membranes in different situations, such as the “sinking raft model”, “leaky-slit” model and “molecular electroporation model” [77]. It is worth mentioning here that these proposed models are not mutually exclusive, which means that both of them may play a role in elucidating certain oncolytic peptides-mediated cancer membrane lysis process. For example, there is likely more than one sequential region in the relatively longer peptides such as LL-37, where the binding modes of actions may alter within the same peptide [69]. Moreover, the mechanism for membrane-active oncolytic peptides-mediated tumor cells death is not entirely caused by plasma membrane disruption, that is, certain peptides can translocate across the membrane and target the intracellular membranous compartments including mitochondrial membrane, endoplasmic reticulum membrane, Golgi membrane and lysosomes membrane [82–88]. However, detailed discussion about these aspects is beyond the scope of our review and remains to be summarized in the future.

2.3. Immune responses elicited by oncolytic peptides

The term “oncolytic” originated in the 1950s and was commonly used to describe the process of oncolytic viruses-mediated tumor cell lysis (oncolysis) [89,90]. Oncolytic viruses have been reported to trigger immunogenic cancer cell death (ICD) and induce systemic anticancer immune responses, which can induce abscopal effects for the treatment of advanced tumors [91,92]. Recently, a growing number of studies have suggested that the anticancer activity of oncolytic peptides not only depends on the membrane lysis effect but also depends on the immune activation events after oncolysis [92] (Fig. 3). LTX-302, a 9-mer cationic peptide derived from the bovine lactoferricin, was reported to

induce subcutaneous xenograft A20 cells lymphomas regression [93]. The results suggested that intratumoral injection of LTX-302 can trigger cancer cell membrane damage and extensive tumor necrosis. Subsequently, the lysed cancer cells would release tumor-associated antigen (TAA), which can be presented by dendritic cells to T cells. In addition, the released damage-associated molecular patterns (DAMPs) such as high mobility group box protein 1 (HMGB1), will promote the antigen uptake and presentation process. The poor treatment effect in nude mice and tumor-rechallenge experiment in wild-type mice further revealed that LTX-302 injection not only induced local oncolytic effect, but also caused a specific and long-term systemic antitumor immunity. LTX-315 (trade name Oncopore™), a de novo designed membrane-active cationic nonapeptide, can also trigger the release of DAMPs (HMGB1 and ATP), promote the secretion of proinflammatory cytokines (e.g., IL1 β , IL6, and IL18), and induce the infiltration of immune cells into solid tumor [94, 95]. Furthermore, a series of studies have shown that LTX-315 is a broad-spectrum oncolytic agent, which can induce ICD in many tumor types including B16 melanomas, MCA205 fibrosarcomas, mesenchymal subcutaneous sarcomas, and pancreatic cancer [96–98]. Significantly, the therapeutic potential of LTX-315 has been validated in human clinical trials, that is, intratumoral injection of LTX-315 has been shown to not only promote the infiltration of CD8⁺ T cells within the tumor, but also expand the T-cell clones in blood [99,100]. It is worth mentioning that, although the oncolytic peptides are expected to act primarily on the tumor cells and trigger ICD, the interactions between various immune cells and oncolytic peptides are also very important for regulating an antitumor immune response, which unfortunately has long been ignored.

2.4. Factors that contribute to the biological activity of oncolytic peptides

Most of oncolytic peptides are derived from AMPs, although more than 20,000 AMPs have been discovered up to now (<http://dramp.cpu-bioinform.org/>), it is worth noting that there are far fewer AMPs present with significant oncolytic activity (<http://crdd.osdd.net/raghava/cancerppd/index.php>). By systematically exploring the structure-activity relationship of oncolytic peptides, not only the biological activity (e.g., anticancer activity, selectivity, stability, bioavailability) of naturally existing oncolytic peptides could be improved, but also more

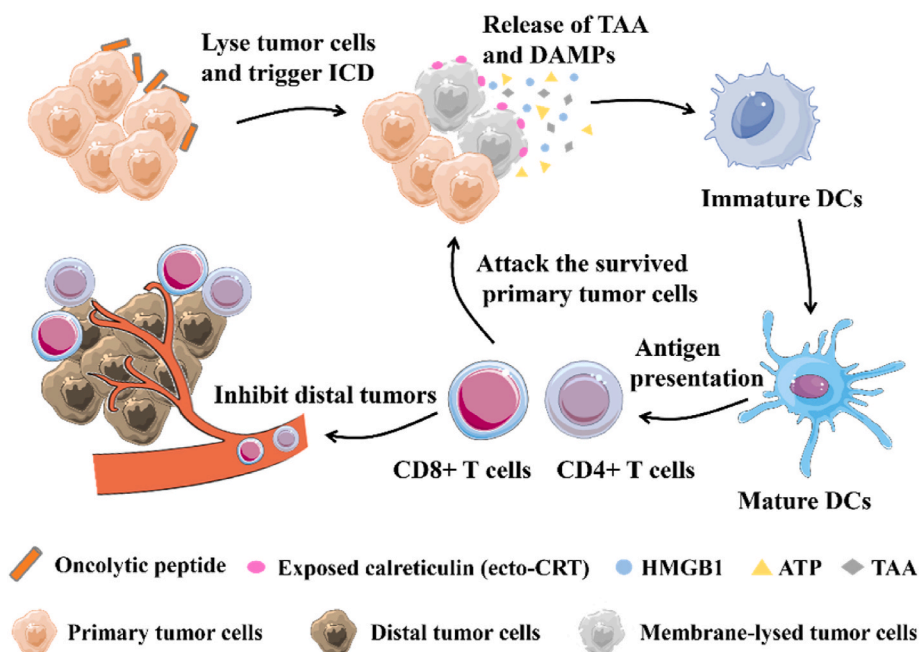


Fig. 3. Schematic overview of the representative immune activation effects of oncolytic peptides-mediated tumor cell lysis.

well-designed synthetic oncolytic peptides and bionic oncolytic polymers are expected to be developed for future clinical applications. Therefore, several important parameters influencing the potential treatment effect of oncolytic peptides would be briefly discussed here.

2.4.1. Positive charge

Cationic oncolytic peptides commonly contain net positive charges varying from 2 to 12 at neutral pH, which is mainly provided by L-arginine residue and L-lysine residue [36,101]. The cationic peptides are likely to induce electrostatic interactions with the negatively charged membrane, which is the major driving force for the adsorption and accumulation of oncolytic peptides on tumor cells [102]. For instance, melittin was reported to lose the oncolytic activity when its C-terminus positively charged residues were replaced by negatively charged residues, and the oncolytic activity would be restored by additional conjugation of a cationic sequence in its C-terminal domain [103]. However, these peptides containing positive charges (e.g., temporin family) may cause severe hemolysis. Considering that the arginine residue showed a stronger interaction with both anionic and zwitterionic membranes than lysine residue, introducing lysine instead of arginine into synthetic oncolytic peptides will reduce the hemolytic effect to some extent [104]. In addition, a high density of positive charges may reduce the blood circulation time and bioavailability of oncolytic peptides, and even cause severe off-target effects [42,105].

2.4.2. Hydrophobicity

Generally, oncolytic peptides contain more than 30% hydrophobic amino acids, which can facilitate the insertion of peptides into the hydrophobic phospholipid bilayers and then cause membrane perturbation [101,106]. Huang & Fei et al. have replaced the alanine residues in the cationic amphipathic peptides with leucine to enhance their hydrophobicity, and the results suggested that their anticancer activity was significantly increased [107,108]. Furthermore, the introduction of fatty acids with C12 to C20 chain lengths to the N-terminus of amphipathic peptides (LVTX-9 and R-lycosin-I) could significantly increase their anticancer activity [109,110]. However, if the peptides are too hydrophobic, they not only tend to self-aggregate in a physiological environment, but also will target indiscriminately to normal cells and cause toxicity [111]. Therefore, the biological activity of the oncolytic peptides is expected to be increased first and then decreased in response to the increasing hydrophobicity.

2.4.3. Peptides length

The peptide length also plays an important role in the practical application of oncolytic peptides, not only affecting its biological activity, but also determining its production complexity and cost. LL-37, a peptide containing 37 amino acids, is a human cathelicidin-derived AMP that can suppress leukemia, gastric cancer, and colon cancer. Ren et al. have found that FK-16, a short fragment of LL-37 containing 16 amino acids (from residues 17 to 32), showed better anticancer activity than its parent peptide LL-37 [112]. Lu et al. have prepared a series of cationic peptides consisting of repetitive sequences, G (IHK)_nI-NH₂ (n = 1~4). As the sequence length increases, although the peptides exhibited higher membranolytic activity in tumor cells, the toxicity to red blood cells and HDFa cells also increased. The results suggested that G (IHK)₃I-NH₂ had the best skeleton length to simultaneously achieve good oncolytic activity and biocompatibility [113].

2.4.4. Secondary structure

Free oncolytic peptides are commonly random coils in the water phase, however, binding with the anionic model lipid bilayers or cancer cells membrane would lead to conformational changes of the peptides into the extended, α -helical or β -sheeted structure [37]. It was widely reported that the secondary structure of oncolytic peptides can further influence the interaction between themselves and the targeted cell membrane. Li et al. found that the helicity of the ABH3 peptide would be

increased after replacing the alanine and glycine with aminoisobutyric acid, and the mutated peptide showed greater membrane permeability and oncolytic activity [114]. Similarly, Womack et al. substituted asparagine with aspartic acid at position 29 of a chicken NK-lysin containing 30 amino acids, the peptide helicity was reduced and its membrane binding affinity was weakened, thereby leading to a decreased anticancer ability [115]. In addition, numerous studies show that incorporation of proline, changing the proline conformation (D-/L-), or adjusting the position of proline will both greatly affect the secondary structure of oncolytic peptides, and further affect the interaction between peptides and tumor cells [116,117].

2.4.5. Targeting units

As we have discussed above, the anticancer activity of natural oncolytic peptides mainly depends on non-nonspecific physical interactions such as electrostatic and hydrophobic interactions. Although the differences between normal cells and cancer cells membrane provide a physical basis for the selectivity of these oncolytic peptides, the imperfect selectivity may still result in off-target toxicity [30]. The incorporation of tumor-specific ligands into oncolytic peptides is an efficient strategy for improving their selectivity and therapeutic effect. For example, EGFR, a receptor overexpressed in a wide spectrum of epithelial-derived cancer cells, has been considered as a tumor-specific target for drug design. Kawakami et al. have developed an "EGFR-lytic hybrid peptide" containing the EGFR binding domain and the positively charged membranolytic domain, which exhibited high selectivity against EGFR-overexpressing tumor cells (e.g., MDA-MB-231 breast cancer, H322 lung cancer, LNCaP prostate cancer, and U251 glioma cells) and could overcome the resistance to EGFR antibody drugs [118]. Similarly, many other tumor-targeting peptides (e.g., transferrin receptor-binding peptide, LTV peptide, gastrin-releasing peptide, amino-terminal fragment of urokinase-type plasminogen activator, and iRGD peptide) were conjugated to different oncolytic peptides to generate the tumor-specific oncolytic peptides displayed better selectivity and anticancer activity [119–122].

2.4.6. Multi-stimulus responsiveness

TME-activatable oncolytic peptides have been designed based on the difference between normal and tumor tissue, which also aim to improve the therapeutic index and reduce toxicity. For example, membrane type-matrix metalloproteinases (MT-MMP), a proteolytic enzyme tethered to the plasma membrane, has been reported to be overexpressed at the tumor invasion border. A MT-MMP cleavable cyclic 25-mer peptide precursor (cycL-25) was synthesized by adding a MT-MMP-sensitive cyclizing linker to the 18-mer oncolytic peptide, within the TME, the oncolytic activity of the peptide would be restored after MT-MMP-mediated cleavage [123]. Results suggested that the cyclic peptide precursor exhibited significant cytotoxicity against highly invasive MDA-MB-435 (MMP-positive) tumor cells at high concentration, whereas normal RBC cells and noninvasive MCF-7 (MMP-negative) cancer cells were resistant to the peptide precursor at high concentration. But it should be noted that, in the MMP-negative cells, the toxicity and hemolysis of cycL-25 precursor were increased instead of being suppressed at low concentration compared to the parent linear oncolytic peptide. This result may be due to the stabilizing effect of cyclization-mediated partial formation of secondary structure, which could reduce the energy requirements for peptides' initial folding and facilitate the membrane disturbance at low peptides concentration. This phenomenon may present a challenge for the *in vivo* application of cyclic peptide precursor due to the potential off-target toxicity. Moreover, a high concentration of lactic acid is secreted within the tumor due to the up-regulated anaerobic glycolysis, which leads to a lower pH in the tumor than in normal tissue. The acidic microenvironment was used as a trigger by Shai et al. to activate the oncolytic peptide, which means that the peptide can only convert to its active form at acidic pH conditions [124]. They replaced the lysine of [D]-K6L9 with histidine (pK_a6) to

produce the pH-sensitive oncolytic peptide [D]-H6L9, which can only be protonated in the TME after intravenous injection, thereby reducing the unwanted systemic toxicity. Zhao et al. have introduced HCO_3^- groups to interact with the peptide's guanidine group of arginine, the peptide can therefore self-assemble into inactive nanoparticles [125]. The HCO_3^- group would be removed when these nanoparticles arrived at the acidic TME, which could result in the disassembly of the nanoparticle, then producing an activated oncolytic peptide nanomachine to lyse both targeted and neighbor cancer cells. Recently, Luo et al. have developed a self-assembling anticancer peptides precursor by computer-aided tools, which could be cleaved and activated only under the acidic TME containing high levels of human kallikrein 2 (hK2) enzymes [126]. The peptides (CRGDKGPDGKAFRRFLGALFKALSHLL, 1–9 disulfide bond) consists of two functional peptide sequences and a cleavable linker: the $\alpha\text{V}\beta 3$ -targeting sequence (iRGD), the acid-responsive oncolytic peptide sequence PTP-7b (FLGALFKALSHLL), and the hK2-responsive proteolytic sequence (GKAFRR). As expected, the peptide nano-assemblies could be delivered to orthotopic prostate tumors via enhanced permeability and retention (EPR) effect and iRGD targeting effect, and then the reactivated PTP-7b would lyse prostate tumor cells without inducing severe systematic toxicity.

2.4.7. Topology

Unlike the classic linear peptides, the membranolytic peptides with more complex architectures including branched and cyclic structures have been recently proven to be more reliable and efficient in cancer therapy. Recently, a linear parent cationic peptide (LLKK)₄, its counterpart 2-arm branched peptide [(LLKK)₂]₂KC and the 4-arm branched peptide [(LLKK)₂]₂KC)₂ have been synthesized by Huang and his collaborators [127]. They found that the 4-arm branched peptide exhibited better anticancer activity than the 2-arm branched peptide, and showed lower cytotoxicity to normal cells than the linear peptide, suggesting that branch modification of oncolytic peptides may increase their selectivity to tumor cells. In addition, Henriques et al. found that although backbone cyclization of tachyplesin I, II, and III (HDPs derived from horseshoe crab) could not improve their anticancer potency, but the cyclized analogs exhibited better serum stability and lower hemolysis than parent tachyplesin peptides [128]. Similarly, a venom-derived cationic peptide was cyclized by Weidong Zhang's team based on the side-chain-retention stapling strategy [129]. The stapled peptide analog showed higher resistance to protease and better selectivity to tumor cells rather than the corresponding linear peptide, furthermore, it could induce a significant oncolytic effect and immune responses within the immunologically "hot" melanoma models.

2.4.8. Other factors

Elucidating the structure-activity relationship can help to develop novel and efficient oncolytic peptides, in addition to the above-mentioned factors, there are still many other structural or physicochemical properties that may affect the bioactivity of oncolytic peptides. For instance, amphiphilicity, referring to the hydrophobic residues and hydrophilic residues that were orderly spatial segregated on the opposite face, also plays an important role in the insertion process of oncolytic peptides into the membrane bilayer [36,130]. Fei et al. have found that the change in amino acid sequence would reduce the peptide amphiphilicity and then weaken its oncolytic activity [108]. Terminal modifications have also been proven to regulate the physiochemical properties and bioactivity of oncolytic peptides. Lu et al. have deleted the C-terminal isoleucine of the parental peptide G (IHK)₃I-NH₂, the obtained peptides exhibited low permeability across the cholesterol-contained membrane, thereby leading to poor anticancer activity [131]. Furthermore, they found that replacing the N-terminal glycine with alanine, valine, glutamate, or lysine could retain the oncolytic activity while reducing the hemolytic activity of G (IHK)₃I-NH₂, which represents a potential method for improving tumor selectivity of the oncolytic peptides [132]. Extensive literature has

shown that replacing the L-amino acids with their D-amino acid enantiomers is another efficient method to optimize the performance of oncolytic peptides, especially the protease stability and selectivity [133]. It is worth mentioning that Papo et al. have designed a diastereomeric peptide composed of lysine and leucine that can directly be used for systemic administration, without needing any targeted motifs or delivery vesicles, and no side effects or mouse death were noted during the 165-day trial period [134]. However, there are also some reports claiming that excess D-amino acid substitutions may reduce the anticancer activity of oncolytic peptides, and even lead to renal toxicity [135,136]. As can be seen, the performance of oncolytic peptides will be affected by multiple factors, including some other factors that may not have been discussed in detail here.

3. The combinatorial application of oncolytic peptides for cancer therapy

3.1. Combination therapy of oncolytic peptides and chemotherapy drugs

Thanks to the unique membrane disruption anticancer mechanism, oncolytic peptides have shown synergistic or additive effects in cancer therapy when combined with other chemotherapy agents (Table 1). As early as 1992, magainin analogs have been shown to enhance the inhibitory effect of cisplatin, etoposide, and doxorubicin against the SCLC cells [137]. Later, Chen et al. revealed that cecropin A was synergistic with 5-fluorouracil and cytarabine in leukemia cells [138]. Hoskin's team and Blancafort's team have found that both NRC-03 peptide and melittin can improve the killing effect of cisplatin and docetaxel against breast cancer cells [103,139]. Similarly, it was reported that θ -defensin analogs can facilitate the uptake of cisplatin and doxorubicin into MDA-MB-231 cells, thereby decreasing the effective dose of these conventional small-molecule drugs [140]. Mastoparan, a cationic peptide derived from wasp venom, was shown to enhance the killing effect of etoposide *in vitro* and improve gemcitabine efficacy *in vivo* [141]. Nisin, a membranolytic peptide produced by *Lactococcus lactis*, has shown a synergistic effect against MCF-7 cells when co-administered with doxorubicin [142].

In addition, Chen et al. have reported two cationic lytic peptides respectively called C8 and C6, which both could be used as the drug carrier for ellipticine, a non-selective anticancer agent [143,144]. The results suggested that C8 and C6 not only promoted the anticancer activity of ellipticine, but also improved the selectivity of ellipticine towards tumor cells rather than normal cells. Subsequently, Chen's group developed another cationic peptide PAH6, which could decrease the IC₅₀ value of doxorubicin against A549 cells and improve penetration of doxorubicin in the A549 3D-spheroid model [145]. Also, since the bioactivity of oncolytic peptides was not affected by resistance mutations, it has proven to be the potential for overcoming doxorubicin chemoresistance [146,147]. Furthermore, Camilio et al. have found that intra-tumoral injection of LTX-315 can significantly improve the therapeutic efficacy of intravenous administration of doxorubicin, including activating antitumor immune response and increasing the infiltration of CD4⁺ and CD8⁺ immune cells within tumor tissue [148]. Notably, compared with the control group, treatment with LTX-315 and doxorubicin could produce a 60–97% increase in median survival when used in combination with surgery. Although this pioneering research demonstrated synergy between LTX-315 and doxorubicin *in vivo*, the administration/dosing scheme was worthy of further discussion. Since LTX-315 was administered by intra-tumoral injection and doxorubicin was injected intravenously, not only arrival time of different drugs toward tumor tissue was difficult to be controlled precisely, but also it is difficult to determine the actual ratio between LTX-315 and doxorubicin within the tumor. In another study, Yang and his collaborators constructed a peptide-based hydrogel containing hybrid melittin (melittin-RADA₃₂) and doxorubicin. The results suggested that injection of the hydrogel not only destroyed some of the localized tumors, but also could

Table 1 (continued)

Oncolytic peptides	Drugs used in combination therapy	Dosing schemes	Tumor cell lines	In vivo models	Curative effects
LTX-315 [154]	Anti-PD-1 antibody, CpG ODN	administered on days 7, 8, and 9 On days 0, 1, and 2, LTX-315 or α PD-1 was administered each morning, and CpG was injected in the afternoon	B16–F10 cells	B16–F10 tumor bearing mice	effect both in local and distant tumors Increase CTLs and helper T cells, reduce Tregs, promote levels of IL-6, TNF- α , and IFN- γ , produce effector memory T cells
LTX-315 [155]	Pembrolizumab or ipilimumab	LTX-315 was injected twice weekly in the 1, 2, and 3 weeks, and antibody was administered in the 1, 4, and every three weeks thereafter	–	Patients with melanoma and TNBC (triple negative breast cancer)	The disease control rate (complete response [CR] +partial response [PR] +stable disease [SD]) was improved
LTX-401 [156]	Anti-CTLA-4 or/and anti-PD-1 mAbs	LTX-401 was injected firstly and antibodies were sequentially injected on days 6, 9 and 12	–	Bilateral subcutaneous MCA205 fibrosarcoma model	Showed distal and proximal effects, acquired immune memory
Melittin-(RADA) ₆ [157]	KN93, PD-1 antibody	Melittin-(RADA) ₆ -KN93 hydrogel was injected on day 7, and anti-PD-1 was injected on days 7, 9, 11, and 13 (in melanoma model)	B16–F10 cells	Subcutaneous B16–F10 melanoma model, H22 hepatoma ascites model	Increase the ratio of M1/M2 macrophage and expression of the PD-L1, improve the cure rate and survival rate
Melittin [158]	Chlorin e6, anti-PD-1	Chlorin e6-Melittin nanocomplex was injected on day 0, light irradiation was received on day 1, then anti-PD-1 was injected every other day for a total of four doses	4T1 cells	4T1 subcutaneous tumor model	Increase the levels of ICD and numbers of mature DCs, improve the abscopal effect and survival rate
LTX-315 [159]	Radiation therapy, anti-CTLA4 and anti-PD1 antibodies	LTX-315 and radiation were delivered on days 0, 1, and 2, and anti-PD1 was injected on days 3, 4, and 5 (in the 4T1 breast cancer model)	TS/A and 4T1 mammary tumor cells	Double and triple TS/A subcutaneous tumor models, M&D-driven breast cancer model	Induce the accumulation of NK cells, enhance the proximal and distal anticancer effect
Melittin [160]	MnO ₂	Simultaneous administration (in the form of MnO ₂ -melittin nanoparticles)	B16, MC38, and MB49 cells	Subcutaneous unilateral and bilateral tumor models, B16–F10 lung metastasis model	Increase cytotoxicity of melittin when combined with chemodynamic therapy, activate STING pathway and promote APC maturation, inhibit metastasis, promote the levels of CXCL10, IFN- γ , IL-6 and TNF- α
LTX-315 [161] (Incubated with cancer cells <i>in vitro</i> to produce cell vaccine)	Surgery, dexamethasone	Immediately after surgery, (oncolytic cell vaccine & dexamethasone) co-loaded hydrogel was injected with a single dose	4T1 cells	4T1 tumor recurrence and metastasis model after partial tumor resection	Promote levels of CD8 ⁺ T cells and M1-type TAMs, reduce MDSCs and TDSFs, inhibit the postoperative recurrence and metastasis

Note: DCs - dendritic cells; NK - natural killer cells; TAMs - tumor-associated macrophages; CTLs - cytotoxic T lymphocytes; M&D - medroxyprogesterone acetate and 7,12-dimethylbenz[*a*]anthracene; APC - antigen presentation cells; MDSCs - myeloid-derived suppressor cells; TDSFs - tumor-derived secreted factors; PD-1 - programmed cell death protein 1; PD-L1 - programmed death-ligand 1; CTLA-4 - cytotoxic T-lymphocyte-associated protein 4.

reverse immune suppression and trigger a durable systemic immune memory [149].

3.2. Combination therapy of oncolytic peptides and biotherapeutic drugs

Recently, immunotherapy has demonstrated a durable and efficient therapeutic efficacy, which provides a potential weapon against advanced or metastatic cancer [162]. However, only a minority of patients with T cell-inflamed “hot” tumors can benefit from cancer immunotherapy, while most of the patients with poor immunogenic tumors and immunosuppressive TME did not respond to the current

immunotherapy [163]. As mentioned above, given the ability of oncolytic peptides to induce antitumor immunity, a growing number of studies have combined oncolytic peptides with biotherapeutic drugs to improve therapeutic outcomes. For instance, the combined intra-tumoral injection of interleukin (IL)-12 and *D*-K₆L₉ peptide could promote the infiltration of NK cells and CD8⁺ T cells within tumor tissue, thereby inducing complete regression of 60–75% tumors and prolonging mouse survival [150]. Sun et al. have reported a fusion protein melittin-MIL-2, which consists of melittin and a mutant Interleukin (IL)-2 [151]. Melittin-MIL-2 showed the ability to remodel the immunosuppressive TME, such as increasing the level of IFN- γ and decreasing

the level of IL-4. The results also showed that intravenous injection of Melittin-MIL-2 could inhibit the lung metastasis of MDA-MB-231-subcutaneous tumor. Although treatment with melittin or doxorubicin can induce ICD and the infiltration of CD8⁺ T cells within tumors, Sun et al. have found that thymocyte selection-associated high mobility group box protein (TOX), a key factor leading to the T cells exhaustion, would also be up-regulated during the treatment process [152]. Therefore, they developed a carrier-free nanoparticle composed of melittin, doxorubicin, and *anti*-TOX small interfering RNA (siTOX), which can not only activate T cell proliferation, but silence TOX expression to restrict CD8⁺ T cell exhaustion.

Recently, immune checkpoint therapy, one of the most promising treatment strategies for cancer patients, has also been extensively used in combination with oncolytic peptides. Zitvogel et al. have found that intra-tumoral injection of LTX-315 would increase the expression of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) on the CTLs and CD4⁺ T-helper cells membrane [153]. As expected, co-administration of LTX-315 and *anti*-CTLA4 monoclonal antibodies (mAbs) showed a strong synergistic effect in combating MCA205 sarcomas, even in the *anti*-CTLA4 mAb-resistant tumors. LTX-315 has also been reported to reduce the PD-L1 expression by acting on the ATP11B-CMTM6-PD-L1 axis in BXP-3, SW1990, and KPC pancreatic cancer cells, which suggested that LTX-315 has good application prospects in combination with PD-1/PD-L1 blockade [98]. Zhong et al. have developed a cocktail therapy based on LTX-315, immunoadjuvant CpG, and *anti*-PD-1 antibody, in which the median survival time was increased by more than 20 days and 2/7 mice were completely cured, indicating that a robust anti-tumor immune response was maintained [154]. Another inspiring result is the enhanced systemic efficacy and response rates have been observed after the combination therapy of LTX-315 and immune checkpoint inhibitors (pembrolizumab or ipilimumab) in humans based on phase I/II clinical study [155]. LTX-401, another oncolytic peptide, also showed significant anticancer ability against primary tumors and distant tumors when combined with *anti*-CTLA-4/*anti*-PD-1 mAbs, and the cured mice exhibited resistance to the tumor re-challenge [156]. Immunosuppressive cells, such as TAMs in the TME, have been widely accepted to be linked with cancer progression, recurrence, and metastasis. Jin et al. have found that KN93, an inhibitor of Ca²⁺/calmodulin-dependent protein kinase II (CAMKII), can convert the TAMs from M2 towards M1 phenotype, thereby promoting the innate and adaptive immunity when used with melittin derivative melittin-(RADA)₆ [157]. Furthermore, given that the combination of KN93 and melittin-(RADA)₆ increased the expression of PD-L1 within the tumor, the PD-1 antibody was co-injected, and then significant synergy has been observed as expected.

3.3. Other oncolytic peptides-based combination therapy

With preclinical or early clinical success of oncolytic peptides-based synergistic cancer therapy, diversified therapeutic methods are being used in combination with oncolytic peptides, in hopes of further improving the treatment strategies and prognosis. For instance, Yang et al. demonstrated that receiving melittin could significantly enhance the treatment effect of chlorin e6-mediated photodynamic therapy (PDT) [158]. Both PDT and oncolysis would increase the levels of ICD and numbers of mature DCs, furthermore, the co-administration of *anti*-PD1 antibody further improved the abscopal effect and prolonged survival in the 4T1-breast cancer model. A synergistic effect has also been revealed between oncolytic peptide and radiotherapy, local radiotherapy and injection of LTX-315 showed better therapeutic effect against the primary HR⁺ TS/A and 4T1 breast tumors compared with monotherapy [159]. When combined with *anti*-CTLA4 antibody, almost complete regression has been observed on the primary tumors, and a significant inhibitory effect also occurred on the untreated distant tumors. Notably, the research also confirmed that the combination of LTX-315 and radiotherapy mainly triggered NK cells-mediated

anti-tumor immunity rather than T cells immunity. Recently, Zhou et al. have demonstrated that Mn²⁺ could improve the anticancer efficiency of melittin, not only because Mn²⁺ can trigger chemodynamic therapy (Mn²⁺ is capable of converting H₂O₂ into •OH within TME and then causing oxidative damage-induced apoptosis), but also because Mn²⁺ is an immunoadjuvant capable of activating cGAS-STING pathway and then enhancing systemic immune response [160]. Surgery is usually the primary choice for many early-stage cancer, however, surgical stress may create an inflammatory microenvironment and thus promote residual cancer cell metastasis to the pre-metastatic niche (PMN). Li et al. prepared an autologous cancer cells-based vaccine (ACCO) by using LTX-315 to induce 4T1 cells lysis, then a ROS-responsive hydrogel was designed for co-delivery of the ACCO vaccine and dexamethasone [161]. They have found that although injection of the ACCO vaccine could increase the level of CD8⁺ T cells within the residual tumor after surgery, the immunosuppressive cells and related cytokines severely interfere with T-cells function. The combination of dexamethasone and ACCO vaccine not only alleviated the inflammation/immunosuppressive microenvironment, but also disrupted the PMN, thereby reducing the risk of postoperative recurrence and metastasis.

4. Delivery strategies for oncolytic peptides

Despite the potent and multi-faceted anticancer ability to fight against drug resistance, tumor heterogeneity, and metastasis, the *in vivo* applications of most of cationic oncolytic peptides are still hindered by their intrinsic properties, such as electrostatic adsorption to plasma proteins, susceptibility to proteases, unsatisfactory tissue distribution, hemolysis and undesired toxicity [31]. Although many efforts have been devoted to optimize the oncolytic peptides by chemical modification or de novo design (as shown in Section 2.4), these strategies cannot be applied to all existing oncolytic peptides, thus developing general strategies for improving their utility in clinical applications is still necessary. Recently, geared by nanotechnology, various nanocarriers have been well-designed for achieving targeted, specific, controlled oncolytic peptides delivery and multimodal cancer therapy, thereby improving therapeutic efficiency. Generally, there are two ways to load the oncolytic peptides onto/into nanocarriers, that is, physical encapsulation and covalent attachment. In this section, we briefly outline these representative nano-delivery strategies and their advantages.

4.1. Physical encapsulation of oncolytic peptides

Based on the physicochemical structure characteristics of oncolytic peptides, it could be conveniently loaded into the nanocarriers via electrostatic interactions, hydrophobic interactions, hydrogen bonding, or other intermolecular interactions. Liposomes are a common nanodelivery system consisting of the phospholipid bilayer, however, it has been a great challenge to load the membranolytic oncolytic peptides without causing leakage of liposomes. In 2008, Wickline et al. have reported a nanoparticle with perfluorocarbon as the core and phospholipids (egg-lecithin & dipalmitoyl-phosphatidylethanolamine) as the outer lipid monolayer [164]. The results suggested that melittin can be stably loaded into the lipid monolayers, which may be due to the hydrophobic and lipophobic perfluorocarbon core that restricts the melittin-mediated membrane destruction (Fig. 4A). Later, the safety and effectiveness of the melittin nanoparticles have been further confirmed in the precancerous lesions and solid tumors models [165]. However, compared to the large-sized melittin-perfluorocarbon nanoparticle (~270 nm), Zhang et al. have prepared a small melittin-loaded monolayer liposome (~20 nm), which is more suitable for the EPR effect. Briefly, a high-density lipoprotein-mimicking α-helical peptide was used to be conjugated with melittin, and the obtained α-hybrid peptide can be deeply incorporated within the phospholipid monolayer (Fig. 4B), then fabricating into a stable nanoparticle (α-melittin-NP) [166]. To improve

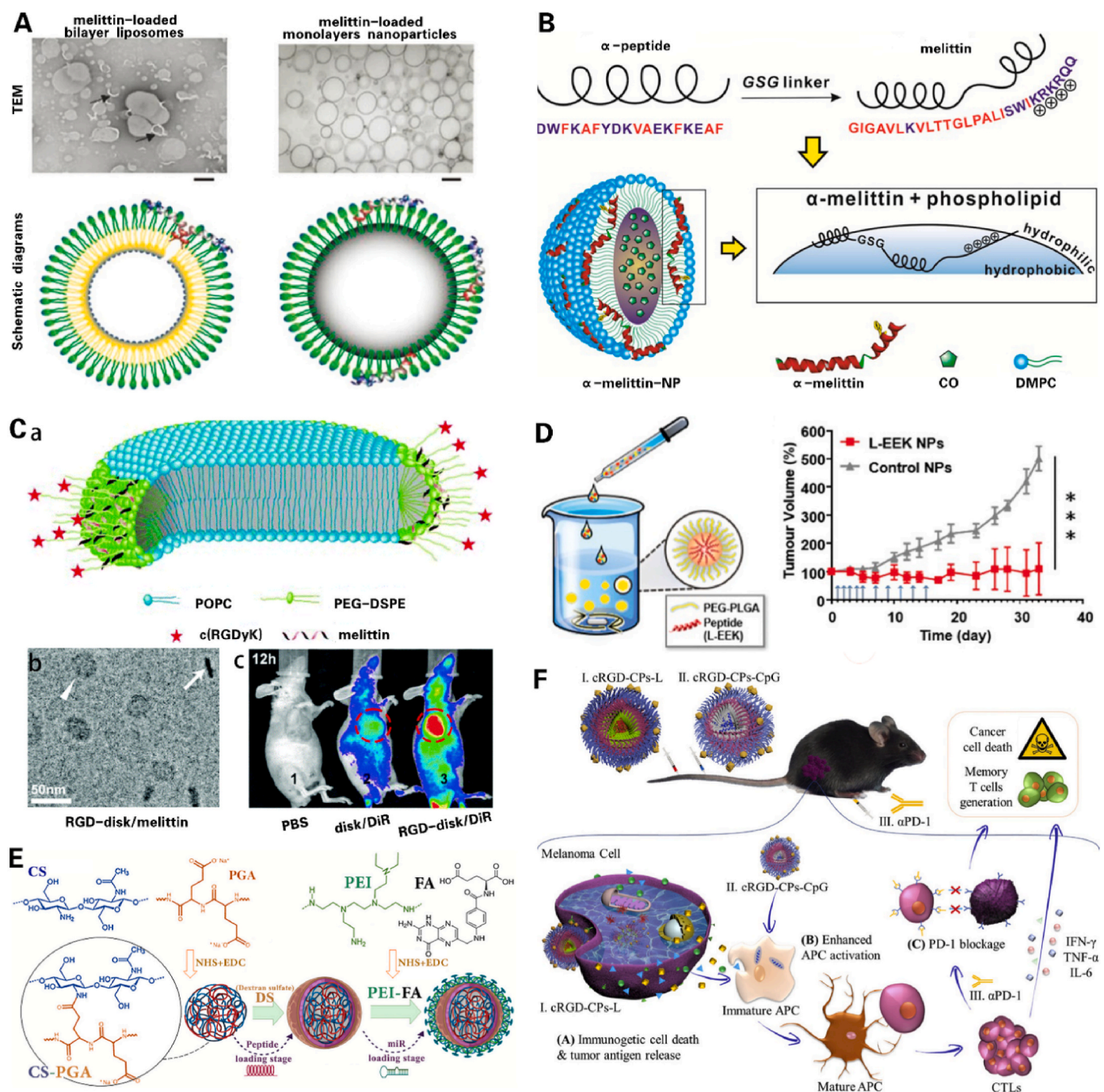


Fig. 4. (A) Transmission electron microscope (TEM) images and schematic diagrams of the melittin-loaded bilayer liposomes as well as melittin-loaded monolayers lipid-perfluorocarbon nanoparticles (Scale bars: 200 nm). Arrows point to the damaged liposome membrane. Adapted with permission from Ref. [164] Copyright 2008 American Chemical Society. (B) Schematic diagrams of the α -helical peptide-melittin hybrid peptide and the corresponding α -melittin-NPs. Adapted with permission from Ref. [166] Copyright 2013 American Chemical Society. (C) a) Schematic diagram and b) TEM images of c(RGDyK) modified melittin-loaded lipid disks; c) Images of tumor-bearing mice at 12 h after injection of 1-PBS, 2-disk/DiR and 3-RGD-disk/DiR (Tumors are indicated with red dotted circles). Reproduced by permission from Ref. [167] Copyright 2016 The Royal Society of Chemistry. (D) Schematic diagram of L-EEK NPs preparation and the tumor volume curves of MDA-MB-231 tumor-bearing mice treated with or without L-EEK NPs, $***p < 0.005$ ($n = 4$). Reproduced by permission from Ref. [168] Copyright 2022 Wiley-VCH. (E) Schematic description of the oncolytic peptides and miR-34a co-loaded multilayer polyelectrolyte nanocarrier. Adapted with permission from Ref. [169] Copyright 2021 Elsevier Ltd. (F) Schematic diagram of cRGD-CPs-L nanoparticles and the combination therapy of cRGD-CPs-L, CpG adjuvant & anti-PD-1 antibody. Adapted with permission from Ref. [170] Copyright 2021 Elsevier Ltd.

tumor targeting ability, a polyethylene glycol (PEG)-stabilized lipid disk has been modified with c(RGDyK) sequence, and the melittin could be adsorbed on the rim of the lipid disk (Fig. 4C) [167]. However, some researchers doubt whether surface adsorption of melittin can effectively avoid its hemolytic effect [166].

Some biocompatible polymers have been developed as oncolytic peptides delivery carriers due to their highly tunable physicochemical properties. For example, a membrane-lytic peptide (called L-EEK) designed by in silico modeling was encapsulated in PEG-PLGA nanoparticles (Fig. 4D), as expected, the prepared L-EEK nanoparticles

showed lower hemolytic activity and good *in vivo* anticancer effect [168]. It is worth noting that complete tumor eradication has been observed in two of four L-EEK nanoparticles-treated mice, which may be due to their strong antitumor effect on both breast epithelial cancer cells and breast epithelial cancer stem cells. Motiei et al. developed a

multilayer polyelectrolyte nanocarrier to realize the co-delivery of oncolytic peptides and microRNA (miR-34a) (Fig. 4E). Firstly, polyglutamic acid grafted chitosan (PGA-CS) has been employed as the inner core to incorporate cationic peptide (LTX-315 or melittin); next, negatively dextran sulfate was used as a complexing agent to stabilize the

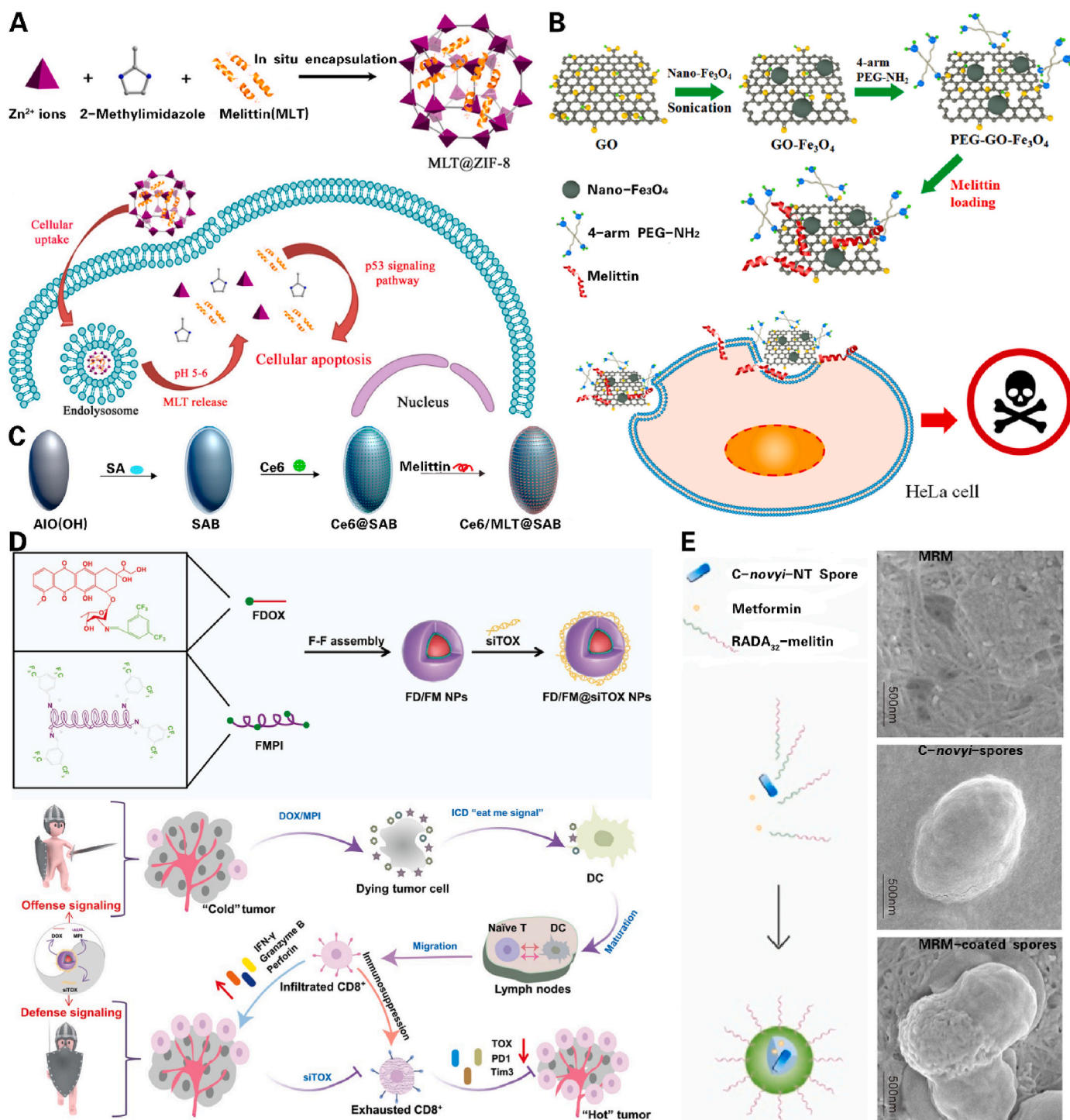


Fig. 5. (A) Fabrication of melittin-loaded ZIF-8 NPs and their application in cancer treatment. Adapted with permission from Ref. [171] Copyright 2018 American Chemical Society. (B) Preparation of melittin-loaded PEG-GO-Fe₃O₄ nanocomposites and the cellular uptake of nanocomposites by HeLa cells. Adapted with permission from Ref. [172] Copyright IOP Publishing. (C) Preparation of Ce6/melittin co-loaded serum albumin/Boehmite nanocomposites. Adapted with permission from Ref. [158] Copyright 2019 American Chemical Society. (D) Preparation of siTOX-coated FD/FM NPs and the manipulation of "offense and defense" signaling by FD/FM@siTOX NPs. Adapted with permission from Ref. [152] Copyright 2022 Wiley-VCH. (E) Preparation of the melittin-RADA₃₂ hydrogel/MET (MRM)-Coated Spores and the TEM images of MRM, *C-novy*-spores, and MRM-coated spores (Scale bars: 500 nm). Reproduced by permission from Ref. [173] Copyright 2022 Elsevier Ltd.

nano-assemblies; finally, folic acid-modified polyethyleneimine/miR-34a complex was added together to form the tumor-targeted nanosystem [169]. This layer-by-layer self-assembly strategy successfully enhances the stability and encapsulation efficiency of cationic membranolytic peptides, and the agents with different mechanisms of action have also been co-loaded to synergistically inhibit tumor growth. Furthermore, a cRGD motif-functionalized, glutathione (GSH)-responsive chimaeric polymersomes has been designed by Zhong et al. to achieve targeted and controlled delivery of LTX-315 (termed as cRGD-CPs-L), in which the cationic LTX-315 was electrostatically adsorbed to the negatively charged poly aspartic acid segment [170]. cRGD-CPs-L could be safely administered via intravenous injection and then deposited within the tumor at 4.8% ID/g, which effectively induced ICD of B16F10 melanoma and resulted in a 30% complete cure rate in combination with immune checkpoint blockade (Fig. 4F). It is worth mentioning that the cRGD functionalized chimaeric polymersomes (cRGD-CPs) reported in this work not only can improve the stability of LTX-315 in blood circulation, but also enhanced its tumor inhibition ability even via intratumoral administration. The results showed that intratumoral injection of free LTX-315 has induced tumor necrosis only on days 1–6, and then rapid tumor recurrence was observed, whereas intratumoral injection of cRGD-CPs-LTX-315 produced sustained tumor suppression.

Since many inorganic materials offer unique cargo-carrying, magnetic, redox, and immunomodulatory properties, several representative oncolytic peptides-loaded organic/inorganic hybrid nanosystems have been developed to improve cancer therapeutic efficacy. For instance, Zeolitic imidazolate framework-8 (ZIF-8), a pH-sensitive metal-organic framework (MOF) consisting of Zn^{2+} and 2-methylimidazole, has been used to deliver melittin for combating U14 tumors *in vivo* (Fig. 5A) [171]. Lin et al. have prepared a PEGylated graphene oxide- Fe_3O_4 (PEG-GO- Fe_3O_4) nanocomposites for melittin delivery (Fig. 5B). The melittin is designed to be shielded by the PEG-GO shell in blood circulation and then transported to the tumor sites under an external magnetic field owing to the Fe_3O_4 component [172]. Boehmite [AlO(OH)] is a pro-oxidant biocompatible material that can accelerate GSH depletion. Yang et al. have developed a nanocarrier by coating boehmite with serum albumin (SA) (Fig. 5C), which was used for the co-delivery of chlorin e6 and melittin [158]. Boehmite not only is a carrier but also can reduce the level of GSH to enhance the PDT effect, and the introduction of melittin not only can promote nanoparticle penetration but synergistically enhance the ICD-associated antitumor immune response.

Recently, carrier-free nanosystems have attracted great attention due to some advantages compared to carrier-based systems, such as facile preparation and high drug loading. Sun et al. have synthesized pH-sensitive prodrugs, fluorinated doxorubicin (FD), and melittin (FM), which can self-assemble to form FD/FM NPs via fluorine interactions and hydrophobic interactions. Then, siTOX (*anti*-TOX small interfering RNA) was further coated on the FD/FM NPs via electrostatic interactions [152]. In the acidic TME, doxorubicin and melittin would be reactivated after the degradation of Schiff base linkage, which can trigger ICD and promote $CD8^+$ T cells infiltration. Moreover, the released siTOX would silence TOX expression to prevent the $CD8^+$ T cells exhaustion, thereby amplifying antitumor immunity (Fig. 5D). Melittin-RADA₃₂, a polypeptide nanofiber previously reported by Yang's group, has been co-deposited with metformin on the *C-novyi*-NT spores by self-assembly (Fig. 5E). It was observed that both the *in vitro* stability and intra-tumoral retention of nano-assemblies have been greatly improved, which could effectively reprogram the tumor immune microenvironment and activate anticancer immunity. In addition, the spore-forming bacteria have only been found in the anaerobic site within tumors rather than normal tissue, suggesting that they can be safely used *in vivo* without inducing undesired infections [173]. Furthermore, Xu et al. designed an amphiphilic lipopeptide composed of dendritic arginine and hydrophobic cholesterol groups. Unlike the nanosystems described above, the amphiphilic lipopeptides can self-assemble to form

hole-punching nanotoxins via supramolecular interactions, without needing any additional components [174]. The nanotoxins could trigger multimodal cancer cells death with 80.3% apoptotic cells (TdT-mediated dUTP nick-end labeling, TUNEL-positive), 97.8% necrotic cells (poly (ADP-ribose) polymerase, PARP-positive) and 71.0% autophagic cells (microtubule-associated protein light chain 3, LC3-positive), which showed great promise for treating multidrug resistant cancer.

While physical encapsulation holds promise for *in vivo* application of oncolytic peptides, a major concern is whether these peptides can maintain their selectivity against tumors once loaded into a delivery system. In one case, the physical encapsulation results in simultaneous decrease of oncolytic activity and hemolytic activity. For example, the oncolytic activity of melittin was partially compromised after physical encapsulation, and meanwhile the hemolytic activity of melittin also significantly decreased, leading to slight increase of tumor selectivity [166,167]. This should be because oncolytic peptides are relatively easy to be released for membrane perforating when the nanoparticles come into contact with highly negative tumor cell membrane [168,175]. Alternately, some other reports claimed that physical encapsulation of oncolytic peptides could reduce the hemolysis without compromising their oncolytic activity, thereby significantly increasing their tumor selectivity. For example, Aronson et al. reported that a kind of lipopeptisome, made of the tumor cell membrane-mimicking lipids, could preferentially fuse with cancer cell membranes and subsequently lead to the direct assimilation of loaded peptides into the membrane lipid bilayer, thereby improving the tumor selectivity [176]. It should be noted that free oncolytic peptides-mediated cell death is mainly due to the disintegration of cell membrane, while after internalization by cancer cells, the oncolytic peptides may act on cytosolic endomembranous organelles [168,170,171]. Moreover, compared to the free oncolytic peptides, the physically encapsulated peptides can selectively accumulate in tumors through either passive targeting (based on EPR effect) or active targeting strategy (e.g. by attachment of targeting moieties), which can further increase the tumor selectivity [167,170].

4.2. Covalent attachment of oncolytic peptides

Compared to physical encapsulation, oncolytic peptides can also be covalently coupled to the polymer chains, which may improve the formulation stability to some extent. Commonly, peptides are chemically bonded to polymer chains via the Michael addition reaction, “Click” reaction, disulfide bond formation, amidation reaction, etc [31].

By introducing the stimuli-sensitive chemical bonds, the oncolytic peptides could be efficiently delivered to tumor tissue using diverse polymer-based vehicles, and then the oncolytic peptides can be released in a controlled manner within TME. Recently, an oil-soluble monomer (termed CDC) was synthesized by using two anhydride groups to modify the terminus of a linear aliphatic chain, which could react with amino groups to form an acid-responsive dynamic amide bond (Fig. 6A). After the oil phase containing CDC has been emulsified under ultrasonication, the water-soluble membranolytic peptide FKLAK could react with CDC at the interface of emulsion droplets to form the polypeptide nanospheres *in situ* [177]. Significantly, the drug-loading efficiency of the nanospheres was as high as 77%, and the FKLAK peptide could be released and reactivated within tumors due to the hydrolysis of the amide bond. Since GSH maintains a high concentration in tumors, oncolytic peptides have been designed to attach to various polymers via disulfide bonds for efficient delivery and controlled release. A negatively charged thiolated amidized glycol chitosan (SA-GCS-SH) was reported by Xu et al. which could serve as a carrier for melittin via electrostatic binding and disulfide cross-linking (Fig. 6B). However, different from the free melittin, the nano-complexes (DSNS) have been found to kill tumor cells by disrupting organelle membranes rather than plasma membranes, this is probably because the encapsulated melittin can only be released under the intracellular acidic pH and high level of GSH [178]. Similarly, another pH/GSH-dual responsive polymer vehicle was

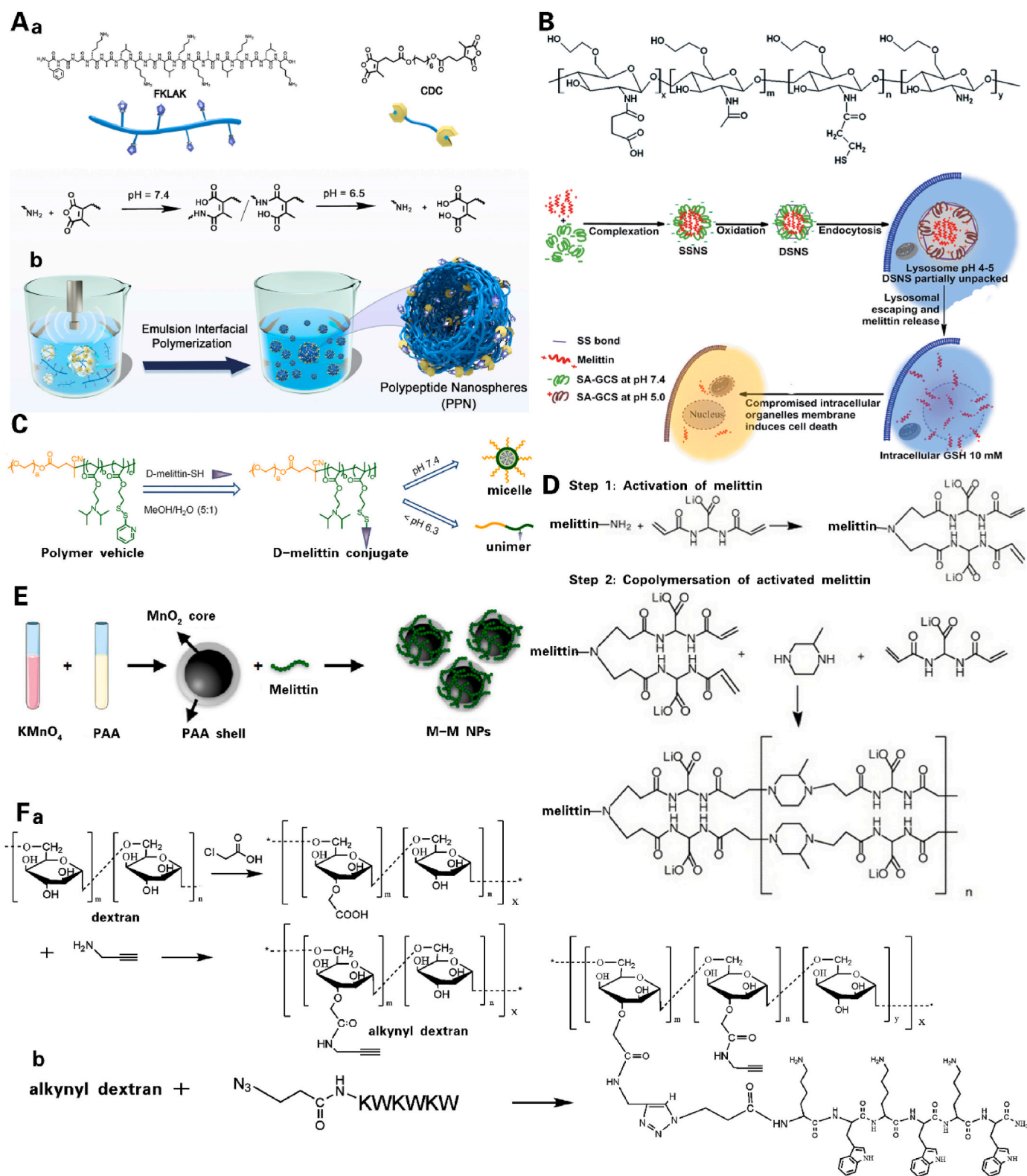


Fig. 6. (A) A) The structures of FKLAK & CDC, the reaction diagram of an amino group and anhydride group at pH 7.4, and the hydrolysis process of the acid-labile amide group at pH 6.5; b) Schematic diagram of polypeptide nanospheres fabricated via emulsion interfacial polymerization. Reproduced by permission from Ref. [177] Copyright 2022 Springer Nature. (B) The chemical structure of SA-GCS-SH and the schematic diagrams of DSNS preparation and its intracellular pathway. Reproduced by permission from Ref. [178] Copyright 2015 Royal Society of Chemistry. (C) Preparation of D-melittin conjugate and its self-assembly behavior at different pH conditions. Reproduced by permission from Ref. [179] Copyright 2021 Elsevier Ltd. (D) The synthesis process of poly (amidoamine)s-melittin conjugates (ISA23-MLT). Adapted with permission from Ref. [180] Copyright 2005 Elsevier Ltd. (E) Schematic diagram of the MnO₂-melittin nanoparticles (M-M NPs) synthesis. Adapted with permission from Ref. [160] Copyright 2022 Elsevier Ltd. (F) a) Synthesis process of alkynyl dextran; b) synthesis process of Dex-(KW)₃ conjugate. Reproduced by permission from Ref. [181] Copyright 2010 American Chemical Society.

prepared by reversible addition-fragmentation chain-transfer (RAFT) polymerization of pyridyl disulfide ethyl methacrylate (PDSEMA) and 2-diisopropylaminoethyl methacrylate (DIPAMA) using poly (ethylene glycol) methyl ether (4-cyano-4-pentanoate dodecyl trithiocarbonate) as chain transfer agents (Fig. 6C). *D*-melittin was then conjugated to the polymer vehicle via a disulfide bond, which could be released due to the protonation of DIPAMA units and cleavage of disulfide bonds after endocytosis [179]. Previous studies have shown that repeat systemic administration of nano-bioactive drugs may trigger a host immune response, to elicit antibodies against the carrier (e.g., anti-PEG antibodies against PEGylated platforms), which resulted in rapid blood clearance and fatal hypersensitivity reaction. It is inspiring to find that substituting the *L*-melittin with *D*-melittin could attenuate the immune response against the loaded cargos, without compromising its biological activity.

Unlike the oncolytic peptides-nanosystems based on dynamic chemical bonds mentioned above, some uncleavable oncolytic peptides-polymer conjugates also showed superior biological activity. ISA23, an uncharged amphoteric poly (amidoamine)s at pH 7.4, would be protonated and undergo a conformation change from coil to stretch at acidic TME. Duncan et al. have synthesized an “activated melittin comonomer” functionalized with two C–C double bonds (Fig. 6D), which was then used to prepare poly (amidoamine)s-melittin conjugates (ISA23-MLT) together with 2-methylpiperazine and bis-acrylamidoacetic acid via hydrogen-transfer polyaddition reaction [180]. The obtained ISA23-MLT has no hemolytic activity at pH 7.4 due to the shielding effect of the ISA23 coil, as expected, the melittin domains would be exposed and allowed to interact with cancer cell membranes at acidic pH. According to Zhou’s work, melittin also has been successfully conjugated to polyacrylic acid (PAA)-modified manganese dioxide (MnO₂) nanoparticle via 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) condensation reaction (Fig. 6E). The MnO₂-melittin nanoparticles showed significant membranolytic activity and cytotoxicity against B16, MC38, and MB49 tumor cells, but were safe to normal cells including antigen presentation cells [160]. This phenomenon may be probably due to the selective exposure of melittin domains within tumor cells caused by the GSH-specific responsiveness of MnO₂ nanoparticles. Moreover, it is worth noting that polyvalent oncolytic peptides-polymer conjugates may have higher binding affinity to cancer cells membrane than that of free oncolytic peptides. Azide-terminated (KW)₃ cationic peptide has been synthesized by Chau and Zhong (Fig. 6F), which was conjugated to alkynyl dextran via Cu(I)-catalyzed “Click” reaction [181]. The thermodynamic analyses suggested that Dex-(KW)₃ conjugate can insert deeply into the core of the membrane due to the entropy-driven effect, while most of the free (KW)₃ can only be adsorbed on the membrane surface due to the enthalpy-driven effect. As expected, the higher local concentration of (KW)₃ peptide bound to dextran scaffold mainly contribute to its increasing membranolytic activity, which also leads to the superior antitumor activity of Dex-(KW)₃ conjugate against PC-3 cells, MCF-7 cells, and even MES-SA/Dx5 multidrug-resistant cells.

5. Advance of oncolytic polymers

Oncolytic peptides have attracted great interest due to their advantages in combating a broad spectrum of cancers and overcoming multidrug resistance, but the practical application has been significantly delayed due to the inherent limitations of peptide drugs. As mentioned above, the oncolytic peptides can be carefully engineered to have a precise sequence, charge, conformation, assembly, and almost any other desired physicochemical/physiological properties in terms of existing technical methods theoretically. To meet the market demands, not only the biocompatibility, stability, targeting ability and oncolytic activity need to be optimized, but the manufacturing scale, production complexity, and cost also cannot be ignored [30]. Oncolytic polymers, as a class of emerging macromolecular agents in the last ten years, have

been defined as the oncolytic peptides-mimicking polymers that can induce tumor cell lysis and has the potential to treat a broad spectrum of tumors. Unlike the traditional polymers that only act as inert excipients or vehicles in the field of drug delivery (as mentioned in Section 4), oncolytic polymers have intrinsic anticancer activity, which can work independently or synergistically with other antineoplastic strategies. In addition, oncolytic polymers exhibit several advantages over natural oncolytic peptides, such as high stability against proteases, high salt tolerance, flexible structure for chemical modification, better compatibility with drug delivery technology, and ease of production as well as preservation [32,41,154,182–185]. In this section, we describe the recent advances in optimizing the therapeutic index of oncolytic polymers, designing TME-activated oncolytic polymers, and developing synergistic therapeutic strategies with oncolytic polymers against cancers.

5.1. Optimization of the bioactivity of the oncolytic polymers

3-alkylpyridinium polymers (3-APS), a natural product derived from the marine sponge *Reniera sarai*, have been reported to possess membranolytic activity against non small cell lung cancer cells, but are also hemolytic and even lethal when given intravenously. To elucidate the structure-activity relationship, Sepčić et al. have synthesized several 3-APS derivatives (APS7, APS8, and APS12-2) (Fig. 7A), and the results showed that the hemolytic activity of 3-APS would be reduced by decreasing the degree of polymerization and carbon chain lengths at the 3-position [186]. A series of methacrylate random copolymers have been designed to mimic the biological activities of oncolytic peptides by Taichman et al. which contain different lengths of cationic side chains and different ratios of hydrophobic-to-hydrophilic repeat units (Fig. 7B). They found that the antitumor activity of copolymers exhibited a significant increasing trend when the ratio of EMA monomer increased or the cationic side chains was extended [187]. However, the increasing ratio of EMA (which means higher hydrophobicity) would also lead to more severe hemolysis, which may be due to the non-specific hydrophobic interaction with the red blood cell membrane. Moreover, it is worth emphasizing that the P-5 copolymer exhibited significant membrane-disrupting ability and cell-killing ability against dormant PC-3 cells and PC-3 spheroids model, while the docetaxel treated-group showed almost no difference from the control group. Recently, several membrane-active anticancer triblock copolymers PEG-PGC_m-PLA_n (PEG, guanidinium-functionalized polycarbonate, and polylactide blocks) have been synthesized via organocatalytic ring-opening polymerization (OROP) [188]. The cationic polycarbonate segment could be prevented from enzymatic degradation because the triblock copolymers were able to self-assemble into micellar nanostructures to achieve stable, long-term blood circulation (Fig. 7C). The results suggested that the copolymers could destroy cancer cell membranes and metabolic process via a non-apoptotic mechanism, which is different from the mode of action of intracellular-targeted small molecule drugs. As expected, the copolymers showed significantly selective killing of human cancer cell lines (BCap37, HepG2, A549, and A431) and even MDR cancer cell lines (Bats-72 and Bads-200), and its anticancer activity would be increased with the increasing degree of polymerization of guanidinium-functionalized polycarbonate block. In addition, intra-tumor injection of the optimized L3/D3 copolymers could not only could inhibit the orthotopic 4T1 tumor growth, but also showed the ability to prevent tumor metastasis.

Cationic poly (L-lysine) (PLL), a kind of poly (α-amino acid)s synthesized by ring-opening polymerization (ROP) of *N*-carboxyanhydrides (NCAs), can kill certain cancer cells by inducing membrane lysis and subsequent mitochondrial damage, but the non-selective toxicity to normal cells still limits its application. Jan et al. have prepared a poly (L-lysine)-*b*-poly (L-threonine) (PLL-*b*-PLT) block co-polypeptide adopting a random coil-β-sheet conformation, which can form 1D fibril assemblies through the intermolecular hydrogen bonding interactions, thereby

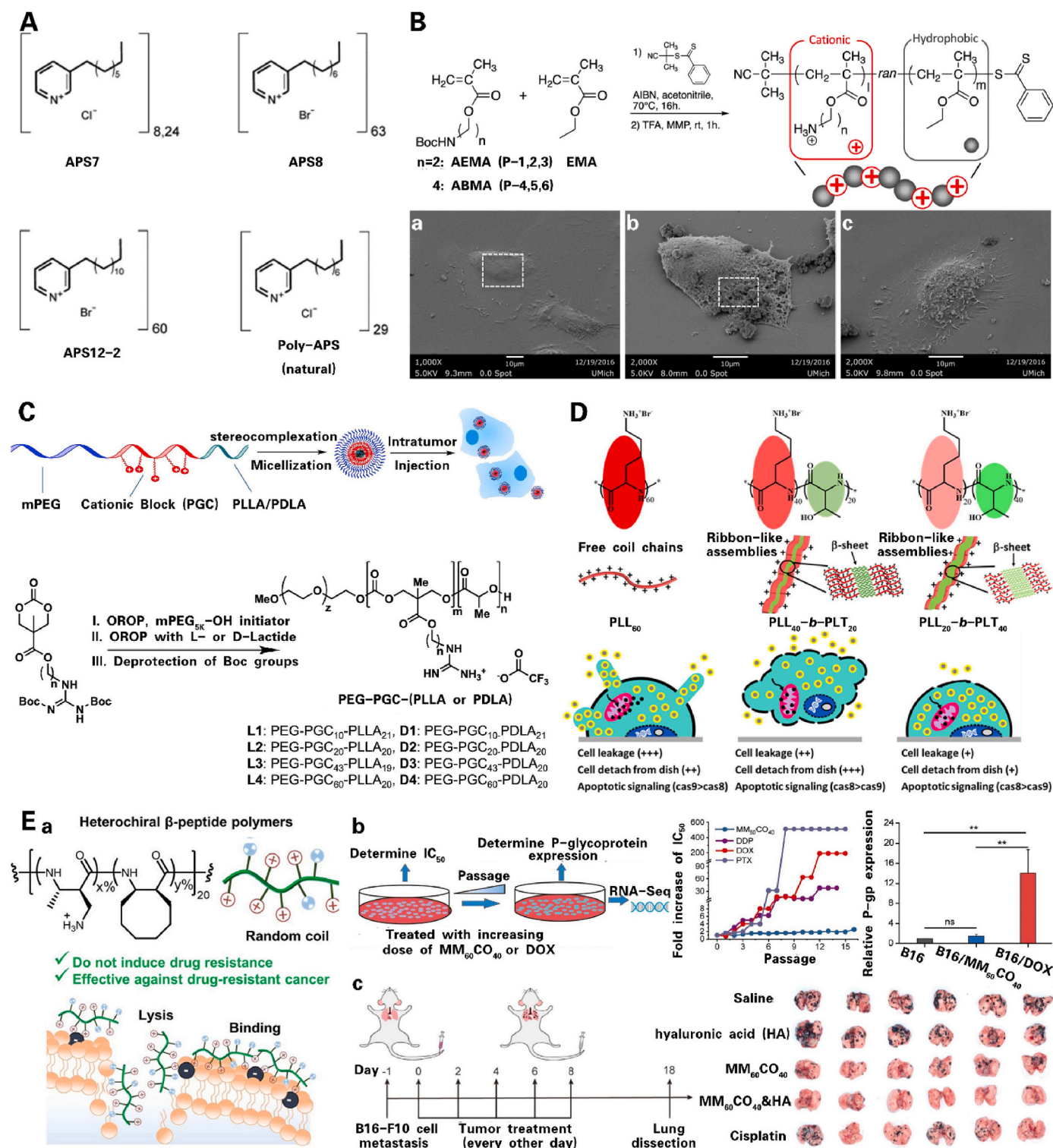


Fig. 7. (A) Chemical structures of the natural 3-APS and its derivatives. Adapted with permission from Ref. [186] Copyright 2012 Elsevier Ltd. (B) Synthesis process of the methacrylate random copolymers (composition of EMA monomer was varied) and SEM images of the a) untreated dormant PC-3 cells and the dormant PC-3 cells treated with b) P-5 copolymer or c) docetaxel. Reproduced by permission from Ref. [187] Copyright 2019 Springer Nature. (C) General synthetic procedure for PEG-PGC_m-PLA_n copolymers and application of the self-assembled nanomicelles in cancer therapy. Reproduced by permission from Ref. [188] Copyright 2019 Elsevier Ltd. (D) Illustration of the chemical structures, self-assembly properties, and anticancer mechanism of the PLL₆₀, PLL₄₀-b-PLT₂₀, and PLL₂₀-b-PLT₄₀. Reproduced by permission from Ref. [189] Copyright 2017 Elsevier Ltd. (E) a) Schematic diagram of design and anticancer mechanism of the heterochiral β-peptide polymers; b) Observation and transcription analysis on the heterochiral β-peptide polymers' insusceptible to antitumor resistance; c) β-peptide polymers suppress the pulmonary metastasis of circulating B16-F10 cells injected via tail vein. Reproduced by permission from Ref. [191] Copyright 2022 American Chemical Society.

shielding the positive charge of the PLL block to some extent and reducing its non-specific adsorption [189] (Fig. 7D). The cell membrane leakage assay revealed that PLL₄₀-b-PLT₂₀ exhibited stronger membranolytic activity than PLL₂₀-b-PLT₄₀, and both of them have lower cytotoxicity to normal cells than PLL₆₀. In the LL2 subcutaneous tumor model, PLL₄₀-b-PLT₂₀-formed fibril assemblies not only could suppress primary tumor growth, but also showed the ability to inhibit lung metastasis and extend tumor-bearing mice survival. Polyaniline (PANI), another cationic polymer with membranolytic activity towards microbial cells, has been used to develop oncolytic polymer [190]. Briefly, an aniline-modified reversible addition-fragmentation chain transfer (RAFT) chain transfer agent (CTA) was synthesized and used to initiate the radical polymerization of acrylic acid to prepare CTA-polyacrylic

acid (CTA-PAA). Then, the nanorod-shaped diblock copolymer (PAN-I-b-PAA) was synthesized by copolymerization of CTA-PAA with aniline monomer to achieve the desired water solubility of the PANI segment. Subsequent cytotoxicity assays revealed that PANI-b-PAA could selectively inhibit the proliferation of HT29 cells at the concentration of 125 $\mu\text{g mL}^{-1}$. It should be mentioned that, although the introduction of polyacrylic acid to the PANI could increase the water-solubility and processability of the copolymer, the oncolytic activity was also reduced due to the negative charge of carboxyl groups.

Lately, a series of heterochiral β -peptide polymers (MM_xCO_y, x: from 50% to 90%) have been prepared to mimic oncolytic peptides via ROP of a monomethyl-substituted cationic β -lactam (MM β) and a cyclo-octyl-substituted hydrophobic β -lactam (CO β) (Fig. 7E-a). The optimized

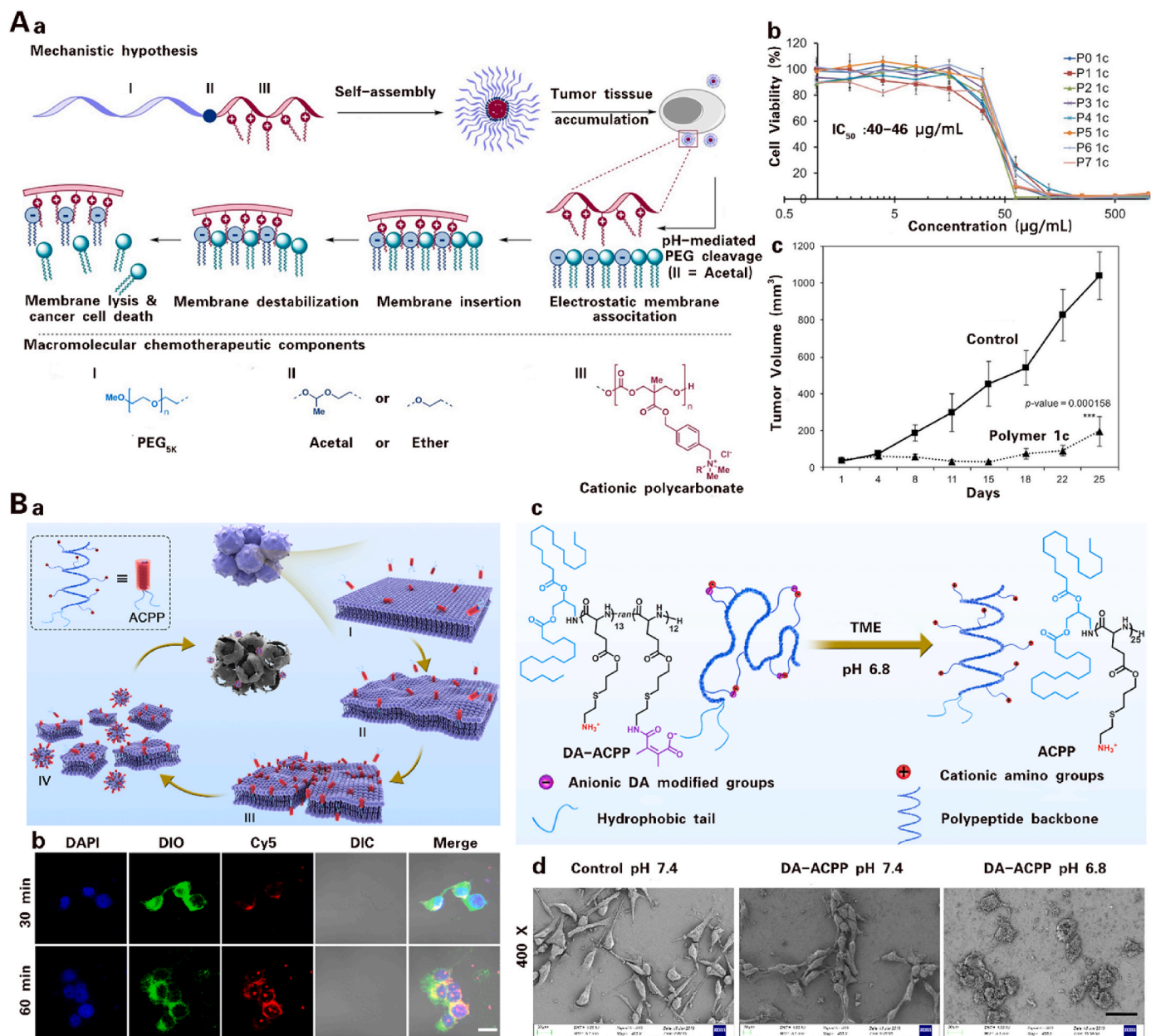


Fig. 8. (A) Design and the underlying mechanism of the pH-activated macromolecular oncolytic agents; (b) The IC₅₀ of polymer 1c against different generations of Hep3B (no drug-resistance has been found); (c) Therapeutic effect of polymer 1c in a human hepatocellular carcinoma PDX model. Reproduced by permission from Ref. [194] Copyright 2018 American Chemical Society. (B) a) Proposed mechanism of the ACPP-mediated membrane-lytic tumor cells necrosis; (b) Localization of ACPP within 4T1 cells after incubation with Cy5-modified ACPP for 30 or 60 min observed by CLSM (nuclei and cell membranes were stained with DAPI and DiO, respectively). Scale bar: 20 μm ; (c) Schematic diagram of the activation of DA-ACPP into ACPP under the acidic TME; (d) SEM images of B16-F10 cells after incubation with DA-ACPP for 12 h at different pH (400 \times magnification, scale bar: 40 μm). Reproduced by permission from Ref. [33] Copyright 2020 Wiley-VCH.

polymers MM₆₀CO₄₀ (degree of polymerization ~20) exhibited a broad-spectrum membrane-disrupting activity towards eight types of drug-sensitive tumor cells and eight types of drug-resistant tumor cells, which at the same time had low hemolytic activity and low cytotoxicity to mammalian cells [191]. Different from the common chemotherapy drugs, continuous exposure of B16 cells to MM₆₀CO₄₀ did not cause drug resistance, the further gene expression levels analysis revealed that MM₆₀CO₄₀-treatment would not induce the enrichment of the MDR-related pathway in B16 cells (Fig. 7E–b). The *in vivo* studies revealed that MM₆₀CO₄₀ (or in the dosage form of MM₆₀CO₄₀ and hyaluronic acid (HA) (MM₆₀CO₄₀+HA)) could not only inhibit both the sensitive B16 solid melanoma, MDR-solid melanomas, and cisplatin (DDP)-resistant A549 lung cancer, but also could prevent the orthotopic MDR-4T1 tumor metastasis and the seeding of circulating B16–F10 melanoma cells (Fig. 7E–c).

5.2. Tumor microenvironment (TME)-activated oncolytic polymers

A class of pH-responsive poly (L-lysine iso-phthalamide) derivatives have been developed more than ten years ago, which can induce membrane damage and shows great application prospects for intracellular drug delivery. However, the IC₅₀ values of these membrane-lytic polymers against HeLa cells were too high to reach >250 μg mL⁻¹, making them unusable as oncolytic polymers to destroy tumors directly [192,193]. To develop a TME-activated macromolecular oncolytic agent, Hedrick and Yang et al. have synthesized a diblock oncolytic polymer consisting of a hydrophilic PEG block and a quaternary ammonium-functionalized polycarbonate block, which is connected by an acid-sensitive acetal linker [194] (Fig. 8A). However, in the subsequent *in vitro* experiments, the acetal linked polymers were observed to have similar cytotoxicity compared to their non-acetal linked control compound, which could be due to the polymers-mediated rapid tumor cells membrane lysis and necrosis taking much less time than hydrolysis of the acetal linker, suggesting that PEG cleavage is not critical for realizing its membrane lytic activity. These results also indicate that the PEG chain might be ineffective to shield the positive charges of oncolytic polymers in these cases, and thus the potential toxicity of these polymers to normal cells or tissues needs to be carefully evaluated. Anyway, because of the membrane disruption mechanism, the optimized polymer 1c was found to effectively kill both the drug-susceptible cells, MDR cells, and cancer stem cells with similar IC₅₀ values, and repeated use of polymer 1c would not induce drug resistance. In addition, tail vein injection of polymer 1c significantly increased tumor necrosis and inhibit tumor growth in the hepatocellular carcinoma patient derived xenograft (PDX) tumor model, with no obvious side effects.

Inspired by natural HDPs, we have reported a polypeptide-based oncolytic polymer (called ACPP) synthesized by ROP and a subsequent “thiol-ene” click reaction, which contained a rigid α-helical peptide backbone, dense positive amino groups at the end of the side chains, and the membrane phospholipid tail-mimicking long hydrophobic alkyl chains [33] (Fig. 8B–a). Most of ACPP was observed to be located on the negative cancer cells membrane after incubation for only 30 min, which led to membrane lysis and rapid necrosis of tumor cells (Fig. 8B–b).

It is worth noting that ACPP showed significant cytotoxicity against all of the tested 12 cell lines including highly metastatic cells and MDR cells, additionally, ACPP exhibited a higher selective index towards tumor cells and higher resistance to proteases when compared with native HDPs (e.g., melittin and temporin B). Prior to systemic injection, the cationic ACPP was converted to the zwitterionic precursor of oncolytic polymer (DA-ACPP) through the modification of ACPP with 2, 3-dimethylmaleic anhydride, which exhibited lower hemolytic and cytotoxic activity against normal cells. As expected, DA-ACPP could be reactivated into ACPP to restore its oncolytic activity in the acidic TME via the pH-triggered breaking of maleic acid amide bonds and the re-exposed positive amino groups at the side chains (Fig. 8B–c&d). Furthermore, a remarkable treatment effect, as well as insignificant

toxicity of DA-ACPP, have been verified in the 4T1 orthotopic subcutaneous tumor model and B16–F10 melanoma lung metastasis model. Subsequently, Yuan et al. synthesized an oncolytic polypeptide (OLPP) with similar chemical structure to ACPP. As expected, OLPP not only showed concentration-dependent membranolytic activity against drug-resistant cancer cell line (CAL27/DDP) *in vitro*, but also could inhibit the tumor growth in mouse models bearing MC38 tumor or Panc02 tumor [195].

A series of polymethacrylates with pH-tunable net positive charges have been synthesized by Yang and her collaborators using dimethyl aminoethyl methacrylate (DMAEMA), hexyl methacrylate (HMA), and methacrylic acid (MAA) as monomers, in which cationic DMAEMA and hydrophobic HMA segments were designed as membrane disrupting domain while anionic MAA was used to provide a zwitterionic protective barrier for prolonging the circulation times, respectively [196]. Although the monomer composition remained unchanged in different polymers, the pKa of the amine group in the DMAEMA segment was observed to be decreased as the degree of polymerization increased. Therefore, at pH 6.8, about 50% amine groups of the DMAEMA segment in polymer M – 14 K were protonated, whereas only 25% amine groups in polymer M – 35 K were protonated, thereby explaining why M – 14 K micelles could dissociate at pH 6.8 and then significantly disrupt 4T1 cells membrane, but M – 35 K showed no activity (Fig. 9A–a). In the 4T1-tumor models, the intratumoral delivery efficiency of M – 14 K (1.01%) is 4.8 times higher than M – 35 K (0.21%) due to the pH-activated interaction between oncolytic polymer with tumor cells, and a single dose of M – 14 K exhibited ability to suppress primary tumor growth and lung metastasis. In another study, they further established a three-dimensional cell spheroid BxPC-3@NIH-3T3 (core: human pancreatic BxPC-3 tumor cells, shell: cancer-associated fibroblasts transformed by NIH-3T3 cells) to mimic pancreatic solid tumor, the results suggested that M – 14 K treatment could significantly kill both the BxPC-3 and activated NIH-3T3 cells [197] (Fig. 9A and b). In the BxPC-3 tumors-bearing nude mice model, M – 14 K showed the ability to eliminate cancer-associated fibroblasts and tumor stroma, thereby making the otherwise sheltered tumor cells easier to be killed by M-14 K. Moreover, different from other stroma remodeling strategies reported in the past, M – 14 K could break the pancreatic cancer stromal barrier and enhance antineoplastic drug penetration, but without promoting tumor metastasis.

In order to improve the sensitivity of the activatable oncolytic polymers in response to the subtle differences between TME and normal tissue, Wang and Xiong et al. have designed a series of oncolytic polymers (named ‘proton transistor’ nanodetergents, pTNTs) composed of a membranolytic block (MB) and a PEG block, which could convert slight changes of pH values into noticeable changes of their membrane disruptive activity. pTNTs were prepared through RAFT polymerization of various methacrylates containing different hydrophobic groups (R) and a methacrylate containing an ionizable tertiary amine group (C6) by using mPEG₁₁₃-CPDB as the macro-CTA, the transition pH (pH_T; the membranolytic activity would be sharply changed at this pH) of which can be finely tuned by adjusting the percentage and species of hydrophobic monomers [198] (Fig. 9B–a). The results showed that benzyl group (Bn)-containing pTNTs (P(C6-Bn₂₀)) could penetrate the membrane with less resistance when compared to hexyl-containing pTNTs (P(C6-H₂₀)), which probably due to the cation-π interaction could make Bn groups exposed on the surface of pTNTs together with the cationic groups (while hexyl groups were mainly located within the hydrophobic core), thereby facilitating Bn groups penetrating into the phospholipids tails domains and causing membrane disruption before the full insertion of polymer into membrane interior (Fig. 9B–b&c). They screened the library of pTNTs and found that P(C6-Bn₂₀) has the highest selectivity (Fig. 9B–d), the cytotoxicity of which would be > 32 times enhanced due to the protonation of the tertiary amine group when pH decreased from 6.9 to 6.8. Subsequently, Panc02_{GFP/mCherry} cells were subcutaneously implanted into the ear of mice, and then the time-dependent

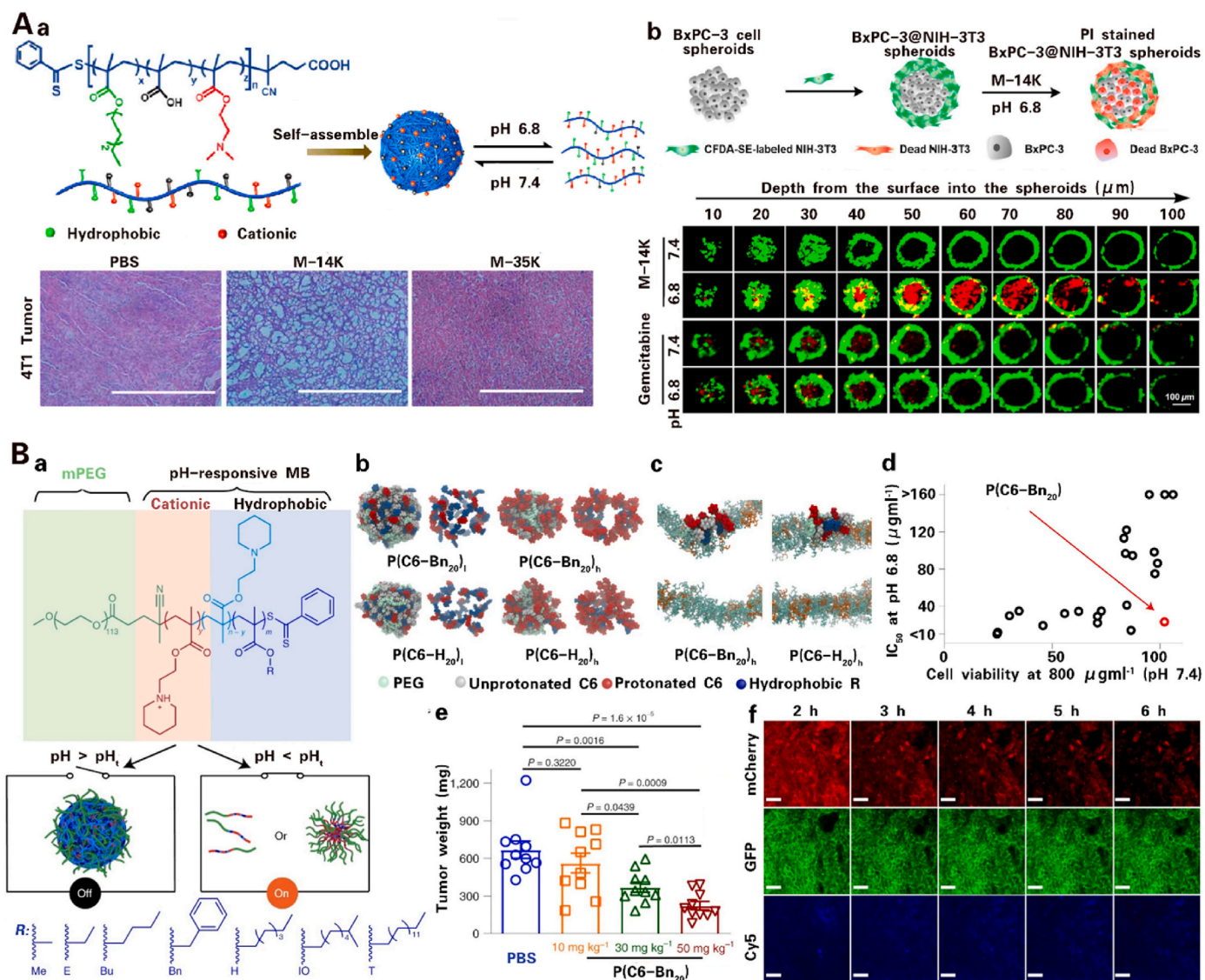


Fig. 9. (A) Schematic illustrations of the chemical structure and self-assembly property of M-14K, and the *in vivo* anticancer effect evaluation of M-14K (scale bar: 400 μm). Reproduced by permission from Ref. [196] Copyright 2020 American Chemical Society; b) Schematic illustration of the M-14K-mediated killing of cancer-associated fibroblasts (activated NIH-3T3) and BxPC-3 pancreatic cancer cells in the three-dimensional BxPC-3@NIH-3T3 spheroid model (NIH-3T3 cells are pre-stained with the CFDA-SE (carboxyfluorescein diacetate succinimidyl ester, green); PI (propidium iodide, red) was used to stain cells with damaged membranes, scale bar: 100 μm). Reproduced by permission from Ref. [197] Copyright 2021 American Chemical Society. (B) a) Schematic illustration of the chemical structure and pH responsiveness of pTNTs. When the pH is higher than the p*H*_t, pTNTs were observed as neutral nanoparticles with inactive membranolytic blocks (MBs) due to the deprotonation of the majority of C6 groups ('OFF' state), while the pH is lower than p*H*_t, the pTNTs transform into free polymer chains or cationic nanoparticles with activated MBs after more C6 groups were protonated ('ON' state). The hydrophobic blocks containing deprotonated C6 and R groups are colored light blue and navy blue, while the positive block containing protonated C6 groups is colored red; b) Atomic molecular dynamics simulations of aggregation state of 16 molecules of P(C6-Bn₂₀)_l, P(C6-Bn₂₀)_h, P(C6-H₂₀)_l and P(C6-H₂₀)_h (the subscript 'l' and 'h' represents 12.5% (low) and 62.5% (high) protonation degree); c) Typical images of the insertion process of P(C6-Bn₂₀)_h and P(C6-H₂₀)_h into the bilayer membrane model; d) The top-performing pTNT was P(C6-Bn₂₀); e) Excised Panc02 tumors weight after the treatment of PBS or different dose of P(C6-Bn₂₀); f) *In vivo* evaluation of the intracellular mCherry leakage after intravenous injection of P(C6-Bn₂₀)-cy5 into Panc02_{GFP/mCherry} tumors-bearing mice, scale bar: 20 μm. Reproduced by permission from Ref. [198] Copyright 2022 Springer Nature.

intracellular mCherry leakage was observed via a bio-imaging system after systematic injection of P(C6-Bn₂₀)-cy5, suggesting that the pTNTs could effectively induce tumor lysis *in vivo* (Fig. 9B–f). As expected, P(C6-Bn₂₀) exhibited good biocompatibility and significant anticancer efficacy in the Panc02 tumor-bearing mice model, CT26 tumor-bearing mice model, B16-F10 lung metastasis model, and PDX breast cancer model.

5.3. Combination cancer therapy with oncolytic polymers

Since the therapeutic window of most chemotherapy agents is

narrow and cancer cells are prone to developing high drug efflux ability, it is urgent to explore methods to reduce the working concentrations of these drugs and reverse the MDR. Yang et al. have developed a quaternary ammonium-functionalized oncolytic polycarbonate to synergize with small molecule chemotherapeutics (Fig. 10A). When combined with only 20 μg mL⁻¹ of oncolytic polymer, the IC₅₀ of doxorubicin for MCF-7 cells would be decreased from about 320 ng mL⁻¹ to 0.5 ng mL⁻¹, suggesting a significant synergistic efficiency [199]. Due to the oncolytic polymer-mediated damage of the plasma membrane, the cellular uptake of doxorubicin into MCF-7 cells was observed to be increased by 2.5 ± 0.5 times after incubation for 2 h. In addition, the polymers also showed

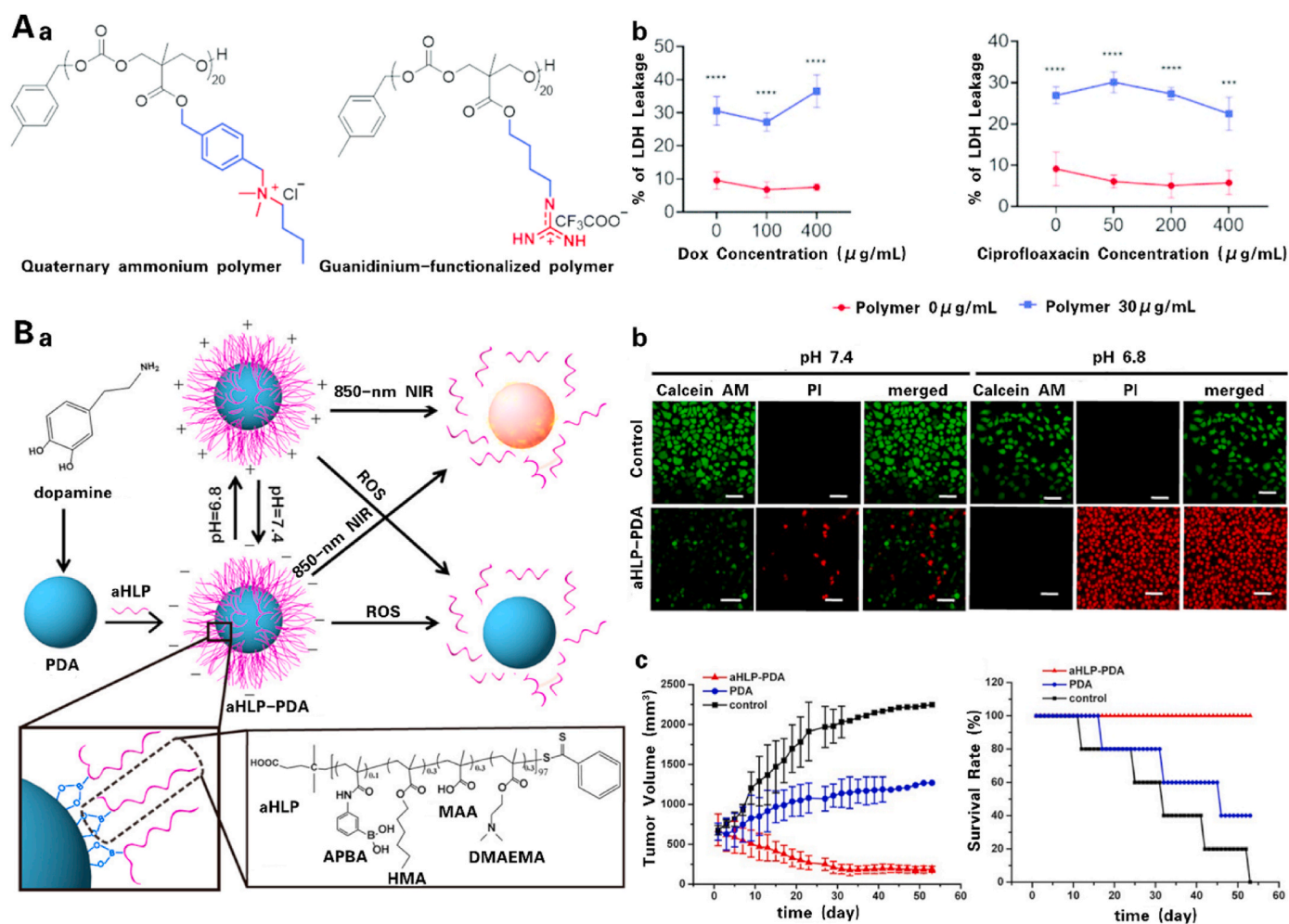


Fig. 10. (A) A Chemical structure of the quaternary ammonium-functionalized oncolytic polymer and the previously reported guanidinium-functionalized polycarbonate; b) The membrane disrupting ability of quaternary ammonium polymer evaluated by lactate dehydrogenase (LDH) leakage assay against MCF-7 ADR cells at different concentrations of Dox or ciprofloxacin. Reproduced by permission from Ref. [199] Copyright 2021 Royal Society of Chemistry. (B) a) The structure and synthesis process of aHLP-PDA nanoparticle, and the schematic diagram of its redox/heat dual-responsive behavior; b) Live/dead (Calcein-AM/PI) staining of the HeLa-TR cells incubated with aHLP-PDA nanoparticle at pH 7.4 or pH 6.8 after 5 min irradiation with a NIR laser at 1 W/cm², scale bar = 50 μm ; c) The tumor volume and survival rate after different treatments evaluated in the 4T1-R tumor-bearing BALB/c mice model. Reproduced by permission from Ref. [200] Copyright 2016 American Chemical Society.

synergistic or additive effects with doxorubicin/paclitaxel/ciprofloxacin against MCF-7 cells, HepG2 cells, and MCF-7 ADR drug resistant cells, respectively. Furthermore, they found that a previously reported guanidinium-functionalized cationic polycarbonate (which can translocate the plasma membrane without causing damage to the membrane) did not exhibit any synergistic effect with doxorubicin, implying that membrane lysis is key for achieving synergistic therapy.

Photothermal therapy (PTT) has attracted extensive attention from researchers due to its spatiotemporal controllability and non-invasiveness. However, heat shock proteins would be overexpressed in the surviving tumor cells after nonlethal photothermal treatment, thereby inducing thermo-tolerance, which means that these cells could acquire tolerance to extreme temperature and drug resistance. Yang et al. have prepared a pH-activated oncolytic polymethacrylate (aHLP) by random copolymerization of 3-methacrylamidophenylboronic acid, hexyl methacrylate, methacrylic acid, and dimethylaminoethyl methacrylate, which exhibited membranolytic activity towards drug-resistant 4T1 (4T1-R) cells and thermo-tolerant HeLa (HeLa-TR) cells at pH 6.8, but showed no activity at pH 7.4 [200]. Then, aHLP was conjugated to the surface of polydopamine (PDA) nanoparticles through the formation of the boronate ester bond, the obtained aHLP-PDA nanoparticle was aimed to achieve synergistic therapy of PTT and oncolytic polymer

(Fig. 10B–a). As expected, the single treatment of PDA nanoparticles with irradiation can effectively kill wild-type HeLa cells at both pH 6.8 and pH 7.4, but most of the HeLa-TR cells were still alive after PTT at either pH. In contrast, after incubation with aHLP-PDA and irradiation with near-infrared (NIR) laser, HeLa-TR cells were significantly inhibited at pH 6.8 due to the release and activation of aHLP (Fig. 10B–b). Moreover, in the 4T1-R tumor-bearing mice model, the intravenous injection of aHLP-PDA could inhibit tumor growth during the entire observation period even after the laser irradiation removal, while the tumors size in the PDA-treated group decreased only after the first light irradiation but continued growing over time, suggesting the oncolytic effect of released aHLP did play an important role in combating drug-resistant tumors (Fig. 10B and c).

6. Summary and prospect

In conclusion, this review summarizes the recent advances on oncolytic peptides at the beginning, including their mechanisms of action to kill tumor cells, the structure-activity relationship, and the emerging oncolytic peptides-based combination cancer therapy strategies. Compared with traditional antineoplastic agents targeting specific metabolic and signaling pathways, most of oncolytic peptides can

rapidly induce plasma membrane perturbation and tumor cell lysis over tens of minutes. The novel targets and killing mechanism of oncolytic peptides endowed it with the ability to overcome tumor penetration barrier, heterogeneity, and multidrug resistance. Next, we summed up the massive efforts devoted to exploring diverse nano-delivery vesicles of oncolytic peptides, which aimed to achieve tumor-targeted controlled release and prevent undesired hemolysis as well as thrombosis. Unfortunately, although oncolytic peptides-based nano-delivery systems can be theoretically well-designed and constructed relying on existing nanotechnology approaches to achieve any desired properties, those nanoparticles with complex structures were still unable to meet the needs for industrial production due to the time-consuming preparation procedures, low production yield, poor reproducibility, and high manufacturing cost [30,201]. Oncolytic polymers, a class of synthetic polymers designed to mimic natural oncolytic peptides, have gradually emerged in recent years, showing great potential as a novel treatment option for malignant tumors. In the last part of this review, the recent advances in oncolytic polymers-mediated cancer therapy have been summarized, and the common strategies used in the design and optimization of oncolytic polymers have also been discussed.

In fact, synthetic polymers have extensively served as an inert component (e.g., drug stabilizer, excipient, smart delivery vehicle, and scaffold) for the treatment of various diseases in the past few decades, which showed a great ability to improve the pharmacokinetics and pharmacodynamics of the functional ingredient [202]. On the contrary, oncolytic polymers, the materials with intrinsic membranolytic activity, can effectively destroy various phenotypes of tumors including MDR and metastatic tumors, without relying on any traditional antineoplastic agents. In addition, synthetic oncolytic polymers can not only be chemically modified via versatile methods and facily produced on a large scale at low cost, but also are compatible with well-established nanotechnology and drug delivery strategies. Due to the many advantages of oncolytic polymers, it may lead to a revolution in cancer therapy in the next few years. However, this is not to say that oncolytic polymers should be required to replace certain antineoplastic agents completely, but it is to say that oncolytic polymers probably serve as a new therapeutic paradigm to complement/improve existing cancer treatment strategies. For example: 1) Since most of oncolytic polymers contain plenty of amino groups, they can be easily post-modified with a diversity of functional units (e.g., proteins, chelations, zwitterions, fluorescent molecules, and targeted agents) via chemical conjugations, which facilitates the regulation of their architectures and expansion of their application. Specifically, modification of amino groups with stimuli-responsive groups leads to construction of stimuli-activable oncolytic polymers with improved therapeutic outcomes and enhanced biosafety [203–205]. However, most of recent researches focus on the design of stimuli-activable oncolytic polymers in response to the acidic pH within TME, the stimuli-activable oncolytic polymers that respond to other internal (e.g., redox state, and various enzymes) and external stimuli (e.g., light, heat, ultrasound and magnetic field) remain to be developed; 2) Due to the broad-spectrum membranolytic activity, oncolytic polymers are able to be designed for inducing lysis of endothelial cells and tumor stromal cells, thereby increasing the vascular permeability and breaking the “dense stromal barrier” to drug delivery [206]; 3) Due to the pore-forming ability on the plasma membrane, oncolytic polymers could break the “cell membranes barrier” and then facilitate both small molecule drugs and macromolecular agents (e.g., nucleic acids and proteins) uptake into the tumor cells or other intra-tumoral cells (e.g., immune cells and stromal cells); 4) PDT or certain cytotoxic drugs are unable to kill cancer cells in the core of solid tumors due to the hypoxic microenvironment, while oncolytic polymers can help to eradicate these hypoxic cells because their antitumor activity would not be affected; 5) Immunotherapy has been shown to deal with various advanced tumors and prolong patient survival, but the response rates to immunotherapy are quite low due to the immunosuppressive TME and immune resistance. In view of the potential of oncolytic

peptides (as described in Section 3.2) in cancer immunotherapy, we have preliminarily demonstrated that oncolytic polymers can also induce ICD of tumor cells, thereby activating the immune response [207]. In addition, oncolytic polymers can be rationally designed to target and lyse intra-tumoral immunosuppressive cells including TAMs, MDSCs, and cancer-associated fibroblasts, which not only can reverse immunosuppression, but also can disrupt the stromal barrier to promote activated immune cells infiltration.

However, as the research of oncolytic polymers is still in its infancy, many doubts and obstacles are remaining to be solved in the future. Firstly, an in-depth understanding of the structure-activity relationships of oncolytic polymers is of high priority before the preclinical trials. Because the interactions between oncolytic polymers and cells membrane were observed to be strongly dependent on the degree of polymerization/backbone length as described above, the controlled polymerization techniques should be optimized to synthesize biodegradable oncolytic polymers with narrower molecular weight distribution, thereby ensuring their physiological activity and avoiding the side effects; Moreover, the influences of comonomer sequence, topological structure, counterions and other factors (such as those listed in Section 2.4) of the oncolytic polymers on their biological activity also need to be carefully studied. Fortunately, instead of screening through millions of different polymers, the introduction of machine learning and artificial intelligence would greatly simplify the process of design & screening of oncolytic polymers, which can reduce the cost and facilitate its further application. Secondly, the underlying action mechanism of oncolytic polymers at the subcellular level/molecular level needs to be explored in depth, for instance, the negatively charged phospholipids-contained endoplasmic reticulum membrane, Golgi membrane, and mitochondrial membrane may also become the potential targets of oncolytic polymers. And various immune cells may also be directly targeted and activated by certain well-designed cationic amphiphilic polymers [208]. In fact, the mechanism of action of oncolytic polymers may not be as simple as disrupting the tumor cells membrane, the largely unknown interactions between oncolytic polymers and non-membrane targets/non-tumor cells need to be further elucidated to ensure their safety and effectiveness *in vivo*. But it is worth noting that studies of the *in vivo* fate and targets of oncolytic polymers usually rely on fluorescence labeling and imaging methods so far, the fluorescent markers and modification sites need to be scrutinized to ensure that the biological activity of oncolytic polymers would not be changed [209]. Besides, to closely approximate the true state of nature, some label-free methods (e.g., surface plasmon resonance, mass spectrometry, bilayer interferometry, and backscattering interferometry) can also aid in the determination of the interactions between oncolytic polymers and the targets without altering the participating components [210]. Thirdly, although all of the oncolytic polymers reported currently have shown the ability to overcome MDR tumors, some studies have shown that tumor cells developed resistance to oncolytic peptides by modulating their plasma membrane components (e.g., anionic glycan), thereby the possibility of oncolytic polymers resistance needs to be examined [211,212]. Fourthly, in order to broaden its application scope, the feasibility and effectiveness of oncolytic polymers in the treatment of some complex tumor models (e.g., glioma/glioblastoma brain tumors) should also be carefully studied. Although there has been some researches showing that oncolytic peptides were able to induce glioma/glioblastoma tumor cells lysis and inhibit growth of subcutaneous glioblastoma tumor [118,213–217], the blood-brain barrier (BBB) remains a major obstacle in the treatment of brain tumors *in vivo*. So far, there has been a controversy about whether the oncolytic peptides/polymers could cross the BBB. For example, although cationic peptide Cypep-1 showed significant anticancer ability against six kinds of glioma cell lines *in vitro*, low concentration of Cypep-1 within the central nervous system indicated that it is hard to pass the BBB [218]. In contrast, some other studies showed that membrane-active peptides (including proline-rich antimicrobial peptides and neutrophilic defensins) can cross the BBB [219,220]. In fact,

most of oncolytic peptides are lipophilic cationic compounds, which are theoretically prone to cross the BBB after certain structural design (e.g., surface charge regulation, targeting ligands modification, introduction of cell-penetrating peptides, etc.) [221–224]. Moreover, the cationic polymers such as poly-*L*-lysines and chitosan, have previously been observed to cross the BBB and used for brain-targeted drug delivery, thus suggesting the broad application prospects of positively-charged oncolytic polymers in the treatment of brain tumors [225]. Finally, although oncolytic polymers have already shown great promise in various mouse models, several fundamental issues still need to be considered before being applied to humans. For example, different administration routes of oncolytic polymers have a great influence on their safety, drug metabolism, and therapeutic efficacy, which should be carefully considered in designing treatment plans. Considering the physicochemical properties and mechanism of action of the oncolytic polymers, developing the oncolytic polymers-based transdermal formulations (e.g., hydrogels and microneedles) seems to be a promising strategy for the local treatment of superficial tumors (e.g., melanoma), thus deserving further exploration in larger trials. More importantly, for the concern of long-term safety, the immunogenicity and genotoxicity of oncolytic polymers and their degradation products also should be carefully investigated on large animals or humans for a relatively long period. Overall, with deepening research, oncolytic polymers are promising to be an integral part of multimodal therapeutic strategies for cancer patients.

CRedit authorship contribution statement

Hanmeng Liu: Investigation, Writing-Original Draft. Wei Shen: Conceptualization, Investigation, Writing-Original Draft, Writing-Review & Editing, Supervision, Funding acquisition. Wanguo Liu: Visualization. Zexin Yang: Data Curation. Dengke Yin: Resources, Funding acquisition. Chunsheng Xiao: Conceptualization, Funding acquisition.

Ethics approval and consent to participate

The present manuscript is a systematic survey based on the review of publicly reported literatures, which did not require ethics review.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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