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Exploiting nanoscale cooperativity for precision medicine

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ABSTRACT

Precise spatiotemporal control of molecular transport is vital to functional physiological systems. Nature evolved to apply macromolecular cooperativity to achieve precision over systemic delivery of important molecules. In drug delivery, conventional nanocarriers employ inert materials and rely on passive accumulation for tissue targeting and diffusion for drug release. Early clinical studies show these nanodrugs have not delivered the anticipated impact on therapy. Inspired by nature, we propose a design principle that incorporates nanoscale cooperativity and phase transition to sense and amplify physiological signals to improve the therapeutic outcome. Using ultra-pH-sensitive (UPS) nanoparticles as an example, we demonstrate how all-or-nothing protonation cooperativity during micelle assembly/disassembly can be exploited to increase dose accumulation and achieve rapid drug release in acidic microenvironments. In a separate study, we show the effectiveness of a single polymer composition to accomplish cytosolic delivery of tumor antigens with activation of stimulator of interferon genes (STING) in lymph node-resident dendritic cells for cancer immunotherapy. Molecular cooperativity is a hallmark of nanobiology that offers a valuable strategy to functionalize nanomedicine systems to achieve precision medicine.

Contents

1. Current success and challenges in therapeutic delivery

Precise molecular delivery in space and time is integral to functional biological systems [1]. In medicine, small molecule drugs are often available to perturb their intended targets; however, poor pharmacokinetics and off-target toxicity limit systemic treatment. Augmenting the physicochemical properties of drug molecules to achieve safety and therapeutic efficacy has driven the field of nanoparticle drug delivery over the past 50 years [2–4].

Several successful examples illustrate the potential of nanotherapeutics, although many more nanosystems have not yet achieved the anticipated impact despite extensive number of publications [5]. As the first FDA approved nanodrug for cancer therapy, liposomal

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formulation of doxorubicin (Doxil®) [6,7] laid the foundation for nano delivery systems inspired by biological carriers [8,9]. Doxil® increased drug accumulation in tumors and avoided cardiac toxicity as with the free drug [10–12]. Drug release in tumors from liposomes, however, is somewhat limited and requires lipase enzymes for carrier degradation [6,13]. Abraxane is a nanoparticle albumin bound (nab) paclitaxel formulation [14] that overcame the adverse side effects from the Cremophor excipient in the original Taxol drug [15–17]. By binding paclitaxel to albumin, the small molecular drug is increased into the nanometer scale, creating a carrier which stabilizes the drug during circulation and increases tumor accumulation after injection. It became one of the few examples of nanodrugs that greatly impacted cancer care (annual sales > $1 billion) [18].

In the 1980s, Jain and Maeda groups independently reported endothelial gaps in tumor vasculature [19] and enhanced permeation and retention (EPR) effect of nanoparticles inside tumors [20]. Extensive research has focused on exploiting the EPR effect to synthesize and evaluate a large number of nanocompositions for tumor-targeted delivery. Among these nanosystems, biodegradable polymeric micelles from poly(ethylene glycol)-*b*-poly(lactic-co-glycolic acid) (PEG-PLGA) were demonstrated by Torchilin and Langer to significantly improve solubilization of hydrophobic drugs with prolonged circulation in blood [21]. While the PEG-PLGA and other nanosystems have become popular in the design of nanomedicine [22–24], these vehicles have not achieved the anticipated impact over existing drug therapy [25]. The clinical setback has invited healthy debates on the validity of EPR effect in human tumors. Increasing evidence suggests that nanoparticle accumulation in tumors is not a passive process through existing endothelial gaps, but rather mediated by active transcytosis [26]. Regardless of active or passive accumulation mechanism, after vascular extravasation, nanoparticles do not remain entrapped indefinitely in tumors, but are subjected to perfusion loss [27,28]. Another limitation of many current delivery systems is associated with leaked cargo outside their target sites ("leaky milk jugs") while only a fraction of payload is released through slow, diffusion-based mechanism in tumors. While our initial inspiration originated from biological carriers as in the case of liposomes, natural systems go beyond physical characteristics (e.g., size, shape) to achieve delivery efficiency. There are a lot to learn from natural delivery systems to fully tap into the promise and excitement surrounding the nanomedicine field.

2. "Cues" from nature

An important biological transport system involves the delivery of oxygen from lungs to tissues to sustain energetic and metabolic homeostasis. Oxygen has low solubility in water and therefore presents a major challenge for delivery. To achieve effective tissue concentrations, hemoglobin and myoglobin proteins are utilized to facilitate oxygen transport and storage. Both proteins bind oxygen through the heme group but display different binding valences. Myoglobin binds a single oxygen molecule [29] whereas hemoglobin can bind four [30]. Oxygen binding to myoglobin is hyperbolic, with high affinity, which assists oxygen storage in muscle tissues [31]. In contrast, oxygen binding to hemoglobin follows a sigmoidal pattern, illustrating molecular cooperativity (Hill coefficient ~ 3) [32,33]. While deoxygenated hemoglobin has a low affinity to oxygen, the affinity increases upon binding to sequential molecules as a result of conformational change [33]. Each binding event leads to a "relaxation" of the heme groups in hemoglobin, allowing a higher affinity for the next molecule. This positive cooperativity enables more efficient loading of oxygen molecules by the hemoglobin in the lung. In the respiring tissue environment, elevated carbon dioxide levels result in a mildly acidic environment upon hydration. The increased acidity stabilizes the hemoglobin in the "tense" deoxygenated state and gradually reduces cooperativity in a pH dependent manner with a non-cooperative state (Hill coefficient ~ 1) at pH 6.0 [34] to facilitate the oxygen release, a phenomenon known as the Bohr effect [32].

Cholesterol transport and absorption represent another highly regulated physiological process that impacts membrane homeostasis and cell signaling. Cholesterol is a hydrophobic tetracyclic molecule that is barely soluble in aqueous environments. Early studies by N. Anitschkow showed rabbits fed on excessive cholesterol diet developed arterial lesions similar to atherosclerosis [35,36], which is corroborated by numerous clinical studies associating high levels of blood cholesterol with myocardial infarcts, stroke, and peripheral vascular disease. Seminal work by Brown and Goldstein uncovered low density lipoprotein (LDL) as a key nanoparticle carrier for cholesterol [37,38]. Each LDL nanoparticle (20–30 nm in diameter) carries approximately 1500 free cholesterol or cholesteryl ester molecules inside the lipid core, which is surrounded by amphiphilic proteins including apolipoprotein B-100 [39]. For entry into a cell, LDL binds to LDL receptors on the cell surface, inducing receptor-mediated endocytosis into clathrin-coated endosomes [37]. Lower endosomal pH causes dissociation of LDL receptors and recycling back to the cell surface, whereas LDL nanoparticles proceed to lysosomes. Within the lysosome, proteins are degraded and cholesteryl ester is converted to cholesterol. Elevated intracellular concentration of cholesterol inhibits the cleavage of sterol regulatory element-binding proteins (SREBPs) that results in downregulation of LDL receptors and reduced cholesterol biosynthesis through a negative feedback loop [40]. Other biological processes that directly impact cholesterol/LDL transport include proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 binds to the LDL receptor and prevents its recycling to the cell surface, thereby reducing cell uptake of LDL nanoparticles [41]. In 2015, the Food and Drug Administration approved alirocumab and evolocumab which inhibit PCSK9 for treatment of hypercholesterolemia by lowering blood LDL particle concentrations [42,43].

Hemoglobin and LDL illustrate the value of multi-valency and molecular cooperativity to improve the precision of molecular transport and delivery. In the first example, hemoglobin binds to four oxygen molecules with successive increase in binding affinity in the oxygenated environment. The binding cooperativity decreases in the mildly acidic respiring tissues. The pH-modulated change in binding cooperativity maximizes the sensing and release efficiency of oxygen delivery between lung and metabolic tissues. In the latter example, lipoproteins first self-assemble into core-shell micelle structures that serve as a soluble nanocarrier for 1500 hydrophobic cholesterol molecules. Sequestration of multiple LDL receptors (50–80% in clathrin-coated pits [39]) drives molecular recognition of LDL nanoparticles for specific and rapid uptake into the cell. Natural processes such as SREBP and PCSK9 activity regulating LDL receptor availability further control the sensing and homeostasis of LDL nanoparticles. Several valuable lessons emerge from these and many other examples. Nature uses cooperativity to maximize delivery efficiency and specificity in response to a physiological signal (e.g. oxygen level, LDL concentration). In addition, phase transition (e.g., conformational changes of hemoglobin, and LDL assembly) can be used as a strategy to non-linearly amplify biological outputs to small changes in physiological signals.

3. Cooperativity as a design principle for nanomedicine

For small molecule drugs, molecular design mainly focuses on the covalent synthesis of complex chemical structures that interact specifically with the intended protein targets. The challenge resides in the stereo-electronic control of small molecule drugs that allow for the most favorable interactions with the binding pocket of the protein or enzyme. Cooperativity among the drug molecules is usually not part of the design consideration. For most small molecule drugs, dose-response relationships follow traditional protein-ligand interactions with hyperbolic trend similar to myoglobin-O_2 binding curves.

Nanomedicine offers a holistic approach that not only targets the intended proteins but also the underlying physiological processes. This system-based approach is more equipped to overcome the complex and heterogeneous biologic barriers over single agent therapy. We postulate that nanoscale cooperativity is not only an opportunity but also a necessity for nanomedicine design to achieve sensitivity and specificity in therapy. Unlike small molecules, nanoscale structures build on a multitude of interacting components to create a system of thermodynamic stability [44]. Electrostatic, hydrogen bonding, and hydrophobic interactions are interwoven in a polyvalent setting to create a responsive, cooperative system that display emergent properties often absent from small molecular behaviors. In these systems, minor environmental shifts can lead to dramatic changes in the thermodynamic properties controlling the system behavior. Overall, this phenomenon allows for the design of all-or-nothing phase transition systems, building over time into new macro-scale functions in therapeutic safety and efficacy.

Conventional nano drug delivery systems consist of inert carriers that rely on a passive diffusion mechanism for drug release and EPR effect for tumor-targeted accumulation. We propose cooperative nanomedicine designs incorporating molecular cooperativity strategies to sense and amplify pathophysiological signals and enable irreversible dose accumulation and instantaneous drug release on site (Fig. 1). By responding to pathophysiologic signatures, the carrier itself has an additional opportunity to engage biological target and exert its own pharmacologic effect. Although significant challenges remain to orchestrate as many functions using as few components as possible (the practice itself is a cooperative process), the payout to acquire a simple but robust system can make a large difference in clinical translation and impact.

4. Ultra-pH sensitive (UPS) nanoparticles: A pH cooperativity system

pH-sensitive drug delivery has been extensively investigated by many labs in the literature [45–54]. These systems incorporate a diverse range of covalent or non-covalent strategies to respond to pH, including surface charge reversal [55], hydrogel swelling [56,57], pH labile conjugation and cleavage of drugs [58–60], non-covalent ionizable systems [61–66], and many more. pH labile systems require breaking of covalent bonds for drug release. Performance of such systems can be limited by the high energy cost of breaking each covalent bond, and cooperative effect between polymer chains is less pronounced. The resulting system usually takes days or weeks for release of therapeutic payloads, and is less responsive to subtle changes in environmental pH [67,68]. Non-covalent pH-sensitive polymers (e.g., PEI, polylysine, chitosan) have been broadly used in drug and gene delivery applications. Most of these systems display broad pH response (e.g., PEI has pH sensitivity from pH 3 to 11 [69]) and may not be able to differentiate small pH changes in pathological indications.

In this section, we use UPS nanoparticles to illustrate how molecular and nanoscale cooperativity can be leveraged to improve the precision of medicine. About a decade ago, our lab established UPS nanoparticles as a new class of tunable pH responsive nanomaterials [70]. Whereas small molecule pH sensors respond to pH in a gradual manner with 10-fold signal changes across 2 pH units, UPS nanoparticles display a binary on/off response (>100-fold) across 0.2 pH units [71,72]. This transistor type of threshold response led to the successful development of fluorescent nanosensors to delineate tumor margins in response to tumor acidic pH, which was well tolerated and allowed visualization of four solid tumor types among 30 patients in a Phase 1 trial [73]. Furthermore, UPS nanoparticles have demonstrated utility in several other applications including endosomal imaging and perturbation [70,74–76], non-invasive PET detection of occult tumors [77], T cell vaccines for cancer immunotherapy [78,79], and tumor-targeted therapeutic delivery [80,81].

4.1. Hallmark of cooperativity: All or nothing protonation of UPS polymers

The emergence of cooperative phase transition in polymeric materials dated back 50 years ago with theoretical predictions by Dušek

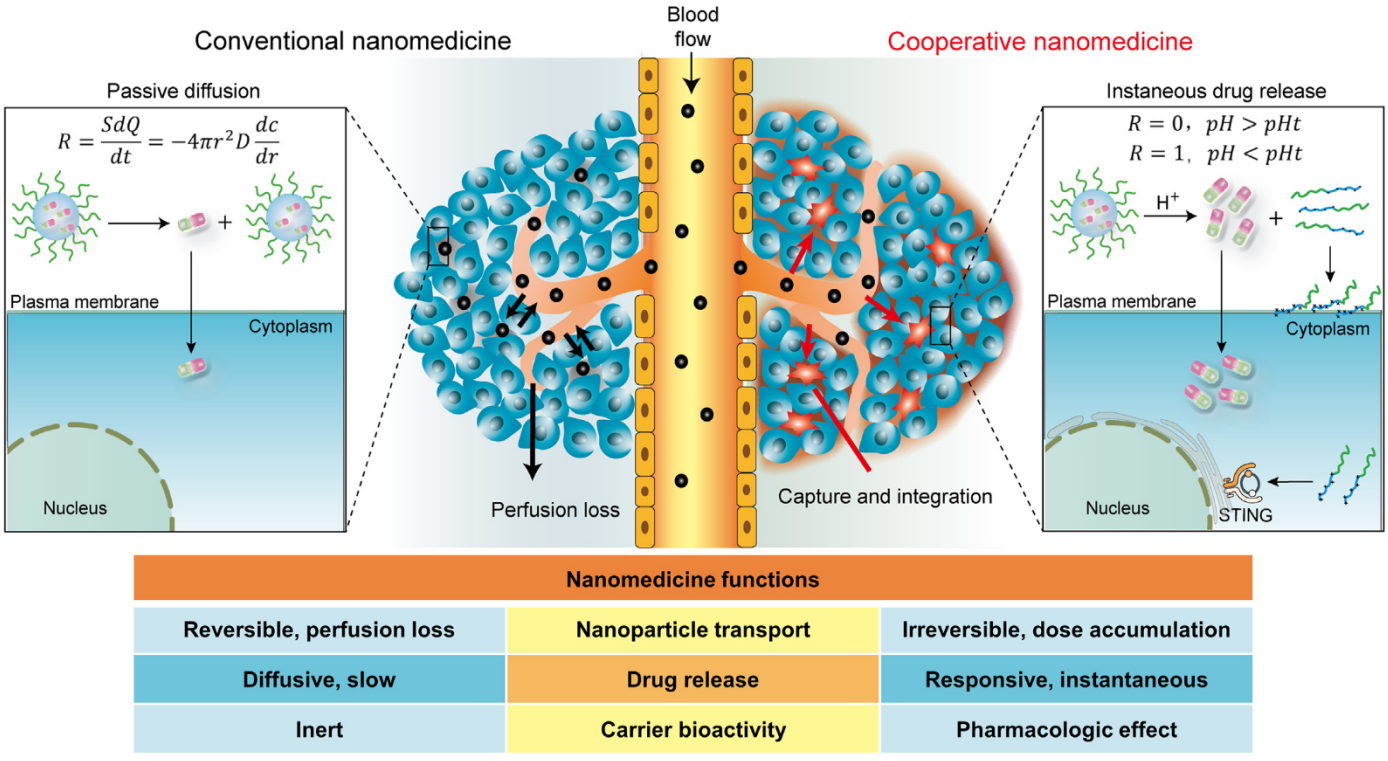

Fig. 1. Cooperative nanomedicine improves payload delivery and drug release over conventional systems. Incorporation of nanoscale cooperativity to sense and amplify physiological signals improves therapeutic efficacy. In selected cases, nanocarriers can also exert their own pharmacological effect.

and Prins in 1969 [82]. In examining the Gaussian behavior of network chains, they postulated the possible coexistence of two polymer phases within a gel network. These metastable phases can lead to discontinuous macroscopic property changes such as volume expansion in a gel network. In his work with thermosensitive gels [83–85], Tanaka observed abrupt swelling behaviors as predicted by Dušek and Prins, and proposed a molecular model with counter balanced forces that are responsible for discontinuous phase transition [86]. Bae reported a sharp pH-responsive aryl sulfonamide polymer gel network with attractive forces in the crystalline state at low pH and repulsive forces from sulfonamide anions in the swollen state at high pH [87,88]. The system was further adopted into non-crosslinked, amphiphilic micelles which rapidly aggregate at low pH, which were designed to enhance passive accumulation of drug carriers within tumors [89].

To consider the molecular force balance which enables the sharp phase transition of UPS polymers, it is useful to consider two polymers of similar structure but exhibiting drastically different pH responsive properties. Poly(ethylene oxide)-*b*-poly(2-(dipropylamino) ethyl methacrylate (PEO-*b*-PDPA) and poly(ethylene oxide)-*b*-poly (2-(dimethylamino)ethyl methacrylate (PEO-*b*-PDMA) differ in the substituents on the tertiary amine sidechains (Fig. 2). When the tertiary amines are protonated at low pH, polymer chains from either polymer will experience electrostatic repulsions because of positively charged ammonium groups. When the pH increases, the propyl substituents in PEO-*b*-PDPA generate sufficient attractive van der Waals interactions to induce micelle self-assembly (~800 polymers per particle [90]). In contrast, PEO-*b*-PDMA lacks sufficient hydrophobicity for self-assembly, and even when fully deprotonated the polymer remains entirely soluble [71].

PEO-*b*-PDMA responds to pH in a gradual, non-cooperative manner (Hill coefficient $n_h \sim 1$). Its protonation behaves like small molecular bases (e.g., dipropyl aminoethanol, DPA, Fig. 2) following the classical Henderson-Hasselbalch scheme that describes the molar fraction of protonated amines (θ_{HN+}) as a function of pH. In contrast, as a result of impending phase transition, PEO-*b*-PDPA is subject to the counteracting forces of the hydrophobic attraction by the neutral side chains or electrostatic repulsion by protonated tertiary amines. These counter forces drive a bimodal distribution of protons between a highly protonated unimer state and a neutral micelle state without intermediates, leading to a remarkable cooperativity in protonation (Hill coefficient $n_h \sim 51$) [71]. This process is similar to the first-order phase transition system such as the melting of crystalline materials where the distribution of materials is in bimodal states (e.g., ice and water, without some form of slurry) [91]. For UPS polymers, the super buffer capacity at the apparent pKa of the polymer is analogous to the latent heat in the crystalline melting scheme where the temperature does not change during phase transition.

This ultrasensitive phase transition response formulates the foundation for the use of the UPS platform in drug delivery, where the micelles will essentially 'dump' their cargo within locales of low pH, such as malignant tumors. From a molecular standpoint, the intermediate state where the pH is close to the pK_a holds interesting insight into the design of new polymer systems with sharp responses. The need to detect and respond to minimal physiological fluctuations poses the requisite of phase transition to achieve molecular cooperativity. Moreover, such phase transition events should engage polyvalent counter-forces whose balance must be shifted by the physiological signal of interest.

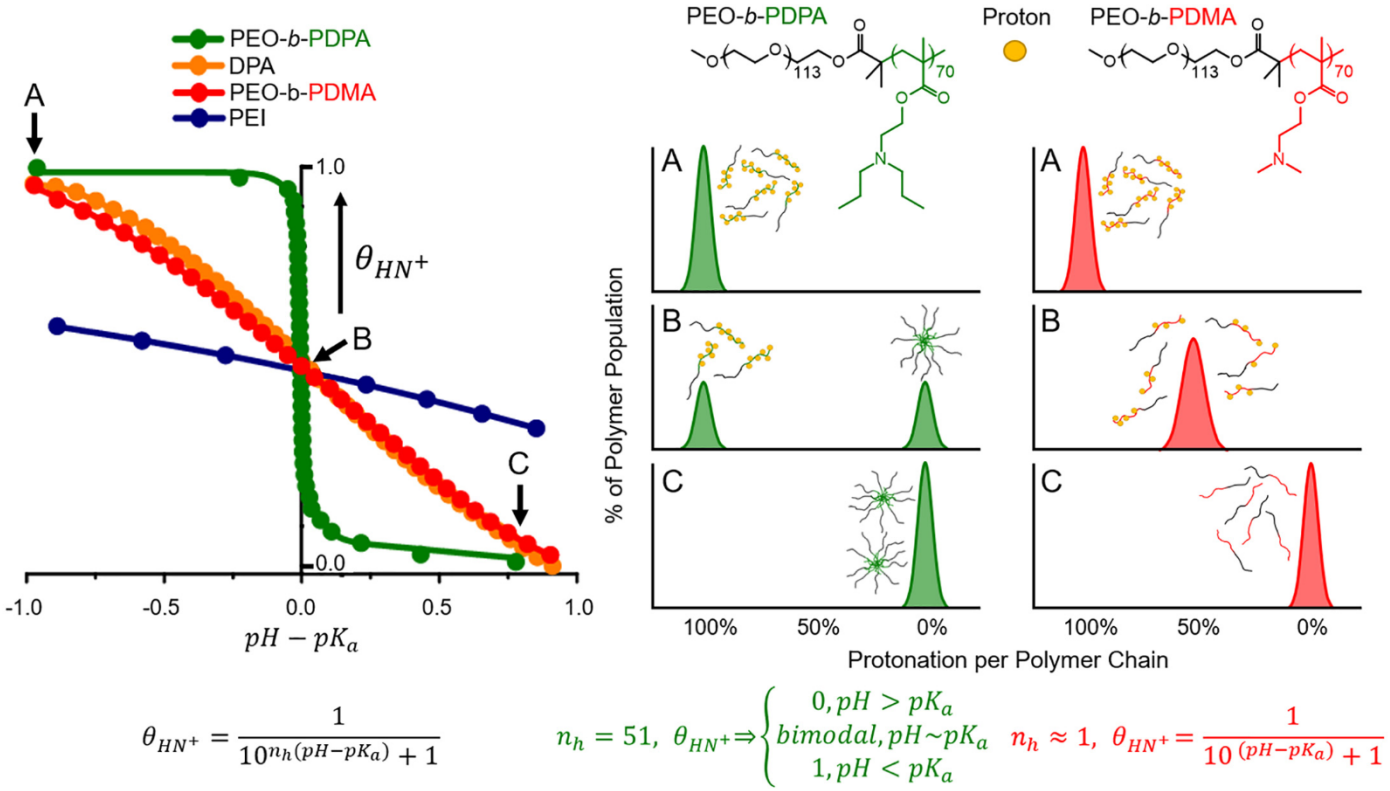

Fig. 2. Ultra-pH sensitive nanoparticles exhibit cooperative, all-or-nothing protonation behavior. UPS polymer PEO-*b*-PDPA protonation response occurs within 0.2 pH units, compared to over 2 pH units for non-UPS polymer PEO-*b*-PDMA and DPA monomer. The distribution of protons among the polymer chains is unimodal in PEO-*b*-PDMA, but bimodal in PEO-*b*-PDPA reflecting the all-or-nothing protonation and phase transition phenomena of UPS polymers. Molar fractions of protonated amines (θ_{HN^+}) can be calculated by the pH and pK_a of the material. Strong cooperativity of UPS nanoparticles ($n_h = 51$) simplifies the protonated species into a binary state (0 or 1). Modified and reprinted with permission from ref. [71]. Copyright 2016 Nature Publishing Group.

4.2. Increased UPS accumulation in tumors driven by pH cooperativity

pH is a fundamental physiologic parameter and becomes dysregulated in a variety of pathologic processes [1,92]. One major motivation for pH-mediated drug delivery is to target acidic tumor microenvironments, where upregulated glycolysis (also known as the Warburg effect) leads to a perpetual state of metabolic acidosis across different types of cancers [93,94]. On the other hand, tumor acidosis is also a transient process depending on oxygen and nutrient levels. At any instant in time, tumor heterogeneity creates an environment with varying levels of acidosis. It is conceivable that gradual pH sensors are subject to noise introduction from spatio-temporal variability as well as low signal amplification intrinsic to the sensor chemistry.

UPS nanoparticles leverage nanoscale cooperativity to achieve a binary detection of solid cancers over the surrounding tissues, which is an emergent macro-scale property integrating spatio-temporal flux of tumor acidosis (Fig. 3). To illustrate the concept, a ^{64}Cu-radiolabeled UPS nanoparticle with a 6.9 transition pH was used to investigate polymer dose accumulation throughout a tumor [77]. Positron emission tomography and autoradiography were used to quantify polymer distribution in the tumor and surrounding tissues over time. After pH-activated micelle disassembly, the polycationic polymers are irreversibly arrested in the protein-bound state or associated with the anionic cell surface, where they are eventually endocytosed for a full capture (Fig. 3a). Transient acidification events in different tumor subzones are sensed by the UPS nanoparticles and radiographic signal is deposited into that microzone. Over time, each transient acidic signal is detected, where UPS nanoparticles disassemble and are captured by the cells, until eventually signal is spread throughout the tumor (Fig. 3b). This “capture and integration” paradigm achieves robust dose accumulation of UPS nanoparticles inside tumors with clear delineation over the surrounding normal tissues, as illustrated in the 73C brain tumor model (Fig. 3c). UPS nanoparticles are able to cross cancer-compromised blood-brain-barriers and accumulate inside tumors. Further comparison of UPS nanoparticles with non-pH sensitive PEG-*b*-poly(lactic acid) (PLA) micelles of similar diameters (25–30 nm) showed significantly increased dose accumulations by the UPS nanoparticles over the PLA micelles (Fig. 3d-f). PLA micelles passively diffuse from the circulation into the tumors which ceases when the concentration of PLA micelles in the tumor equals the (decreasing) concentration in blood. In contrast, dissolution of the UPS micelles and removal of the unimers by proteins and cellular components ensures the concentration gradient between the blood and tumor always favors micelle infiltration into tumors from circulation. Through capture-and-integration, UPS nanoparticles achieved 4-fold higher tumor accumulation than PLA micelles and exhibited strong tumor specificity with little accumulation in surrounding normal tissues.

This phase transition mechanism of UPS accumulation in response to tumor pH led to PET imaging of a variety of occult tumor nodules (10–20 mm^3) in the brain, head/neck and breast tissues with dramatic increase in cancer specificity over small molecular tracers (e.g., ^{18}FDG, ^{11}C methionine). It also provides the foundation of using UPS nanoparticles for therapeutic delivery to solid cancers (**Section 5**).

4.3. pH-triggered drug release

Along with cooperative micelle disassembly, release of encapsulated agents occurs upon environment acidification. We investigated UPS and PLA micelles for their release kinetics of a hydrophobic triptolide prodrug (Fig. 4) [80]. At blood pH (7.4), the release of triptolide prodrug from UPS or PLA micelles is slow and driven by passive diffusion. Less than 20% of the prodrug was released over 24 h. When the environment pH is acidified to 5.0, rapid and complete drug release occurs within 2 h from the UPS system, whereas PLA micelles maintain a slow release rate independent of pH.

The UPS library, with its unique all-or-nothing phase transition behavior and rapid release profile in acidic environments, has drawn the attention of other groups in drug and gene delivery. By dual release of platinum prodrug and a colony stimulating factor 1 receptor inhibitor, Wang and coworkers show UPS nanocarriers induced apoptosis of tumor cells and depleted tumor associated macrophages to achieve therapeutic synergy [95]. Small interfering RNA (siRNA) holds great potential in the silencing of target genes for therapeutic benefit. UPS nanoparticles were used for siRNA delivery with cell penetrating peptides [96] or oligoarginine peptides [97] for enhanced endosomal disruption or with mitoxantrone prodrug [98] as a combination strategy for gene silencing and chemotherapy. Chen et al. reported UPS nanoparticles can be further functionalized with a reactive oxygen species (ROS)-responsive polyprodrug co-delivered with β-lapachone [99]. Inside tumors, β-lapachone and polyprodrug were released, leading to ROS generation. Then, doxorubicin was released from the polyprodrug, ultimately improving the specificity of delivery within tumors. In each of these cases, the UPS nanoparticle system lends rapid release and time-integrated dose capturing to boost antitumor efficacy.

5. Tumor targeted immune reprogramming by UPS nanodrug

Developed by AstraZeneca, AZD3965 (hereafter referred to as AZD) is a small molecule drug that inhibits lactic acid export by cancer cells through monocarboxylate transporter 1 [100]. The current route of oral administration leads to high systemic concentrations of the drug, causing dose limiting side effects in the eyes and hearts of patients [101]. Encapsulation of AZD into UPS nanoparticles allowed us to exploit the cooperative properties in phase transition, capture and integration, and rapid release to improve the drug efficacy at reduced dose (Fig. 5) [81].

After nanoparticle formulation, the AZD drug remains stably loaded in the micelle at pH 7.4 (Fig. 5b). Upon acidification to pH 6.0, the AZD drug is instantaneously released, achieving near 100% release within minutes. Consistent with cooperative protonation and micelle disassembly (**Section 4.1**), AZD release follows an all-or-nothing release phenotype with a designated pH transition of 6.1, the apparent pKa of the specific UPS polymer (Fig. 5c).

Through UPS delivery, the pharmacokinetics and biodistribution of AZD is significantly enhanced compared to the oral administration of the free drug. Intravenous injection of AZD-UPS NPs at 50-fold lower dose from oral administration showed approximately two-fold higher tumor accumulation (Fig. 5d). Furthermore, lower drug concentration in pivotal organs by AZD-UPS NPs resulted in less side effects of AZD as indicated by low cardiac troponin levels in blood. For tumor treatment, the AZD-UPS NP achieved tumor growth inhibition of B16F10 melanoma tumors but showed significant synergy in combination with checkpoint blockade of PD-1 (Fig. 5e-f). Immune profiling of tumor samples showed increased infiltration of antigen-specific T cells and less exhaustive biomarkers (PD1$^+$Tim3$^+$) on these T cells. These studies demonstrate AZD-UPS nanodrug offers a safe and effective reprogramming of tumor microenvironment for improved cancer immunotherapy.

6. STING-activating nanoparticle vaccine for T cell therapy of cancer

Generation of tumor-specific T cells is critical for cancer immunotherapy, which is rapidly emerging as a powerful paradigm for cancer care [102–104]. The spatiotemporal control of antigen transport to the secondary lymphoid organs, cytosolic delivery and cross-presentation in antigen-presenting cells in coordination with innate stimulation are essential to achieve a robust tumor-specific T-cell response (Fig. 6a) [78]. Inspired by ‘proton sponge’ polymers for cytosolic delivery of biologics [105] and the small NP size for lymph node targeting, we screened the library of UPS copolymers to evaluate their abilities in generating a

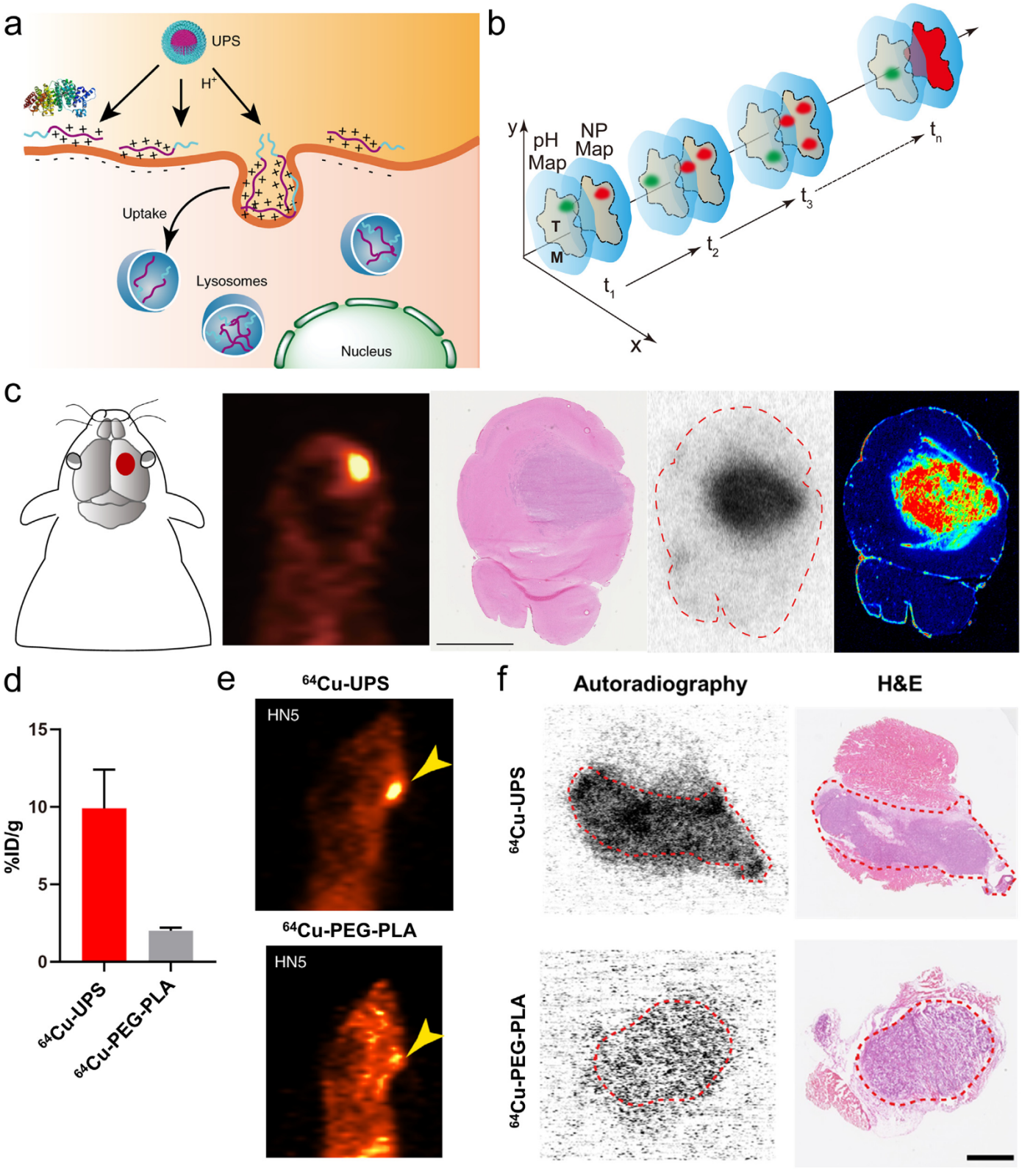

Fig. 3. Nanoscale cooperativity drives UPS polymer accumulation in tumors. (a) The UPS micelles disassemble upon entry into an acidic tumor microenvironment, leading to irreversible activation. (b) Although tumor acidotic processes are transient in nature, the capture of dissociated polycationic polymers over time integrates into a homogenous distribution in tumors. (c) UPS polymers conjugated with either ^{64}Cu (PET image in second panel and autoradiography in fourth panel) or indocyanine green (fluorescent image, fifth panel) were injected intravenously and showed broad distribution throughout the tumor. (d-f) Through capture and integration, UPS nanoparticles achieved four-fold higher tumor accumulation and lower off-target accumulation than PEG-PLA micelles in a HN5 head and neck tumor model. Modified and reprinted with permission from ref. [77]. Copyright 2020 Nature Publishing Group.

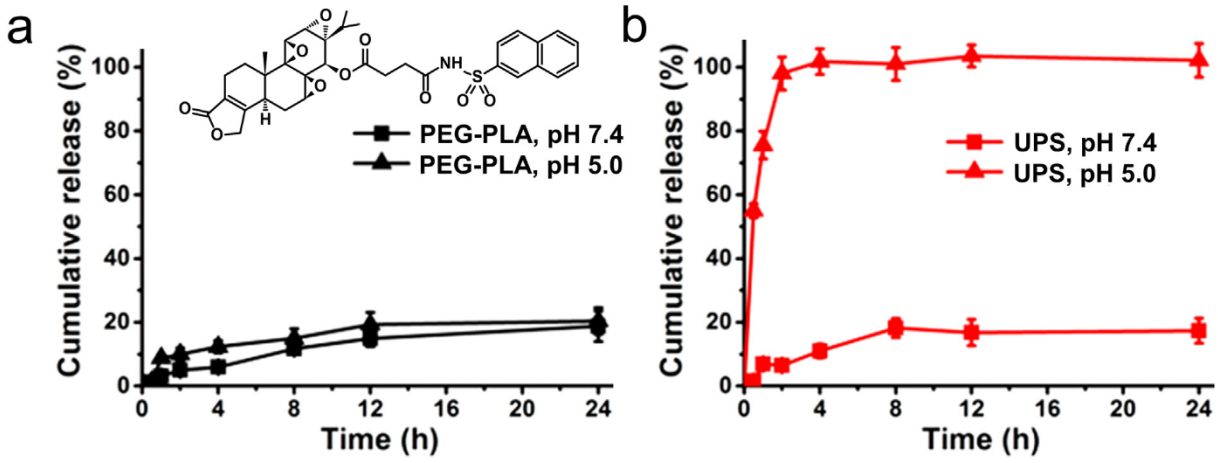

Fig. 4. UPS micelles enable pH-triggered drug release in contrast to slow release from PLA micelles. (a) Release of a representative triptolide prodrug from PLA micelles is slow and not pH dependent. (b) UPS nanoparticles rapidly release the drug within hours of acidification. Modified and reprinted with permission from ref. [80]. Copyright 2019 American Chemical Society.

cytotoxic T lymphocyte response [78,106]. We discovered a minimalist nanovaccine by simple mixture of an antigen with a synthetic polymeric nanoparticle, PC7A NP, which generated a highly specific T-cell response in vivo with low systemic cytokine expression.

Mechanistically, PC7A NP achieves efficient cytosolic delivery of tumor antigens to antigen-presenting cells in draining lymph nodes after subcutaneous administration (Fig. 6b), leading to increased surface antigen presentation (Fig. 6c). More importantly, the PC7A polymer is able to activate the stimulator of interferon genes (STING) to induce downstream production of type I interferons (Fig. 6d), enabling the generation of all necessary signals for T cell activation. In a human papilloma virus-E6/E7 tumor model, TC-1, we demonstrated that PC7A vaccination enables effective control over tumor growth, leading to long term survival of 60% of mice. *Re*-challenge of these tumor-free animals with TC-1 cells led to complete protection against tumor growth, suggesting the generation of long-term antitumor immune memory. The vaccine synergizes with PD-1 checkpoint blockade therapy to achieve complete cures of all treated mice (Fig. 6e, f). The ability of PC7A to serve as a delivery system and innate immune activator allows a single polymer composition to cooperatively solve all of the spatio-temporal requirements for safe and efficacious T cell production in cancer immunotherapy.

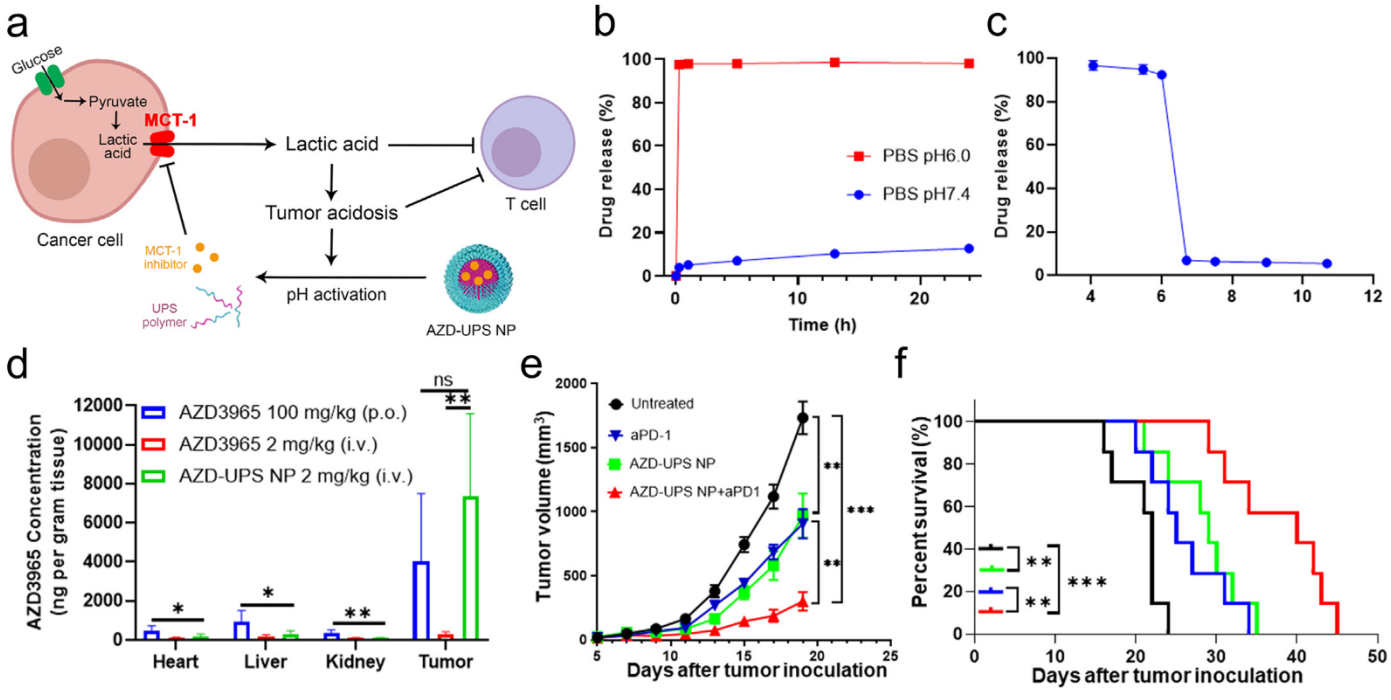

Fig. 5. UPS nanoparticle delivery of AZD drug improves cancer immunotherapy. (a) AZD-UPS nanodrug targets AZD delivery to locations of high MCT-1 activity to inhibit acidosis-mediated suppression of T cells. (b) UPS micelles exhibit 'dose dumping' of AZD drug in low pH environments. (c) Drug release after 15 min resembles pH mediated all-or-nothing disassembly of UPS polymers. The transition pH of the specific UPS polymer is 6.1 in this study. (d) UPS delivery of AZD allowed higher tumor accumulation but lower distribution in pivotal organs at 50-fold less dose compared to oral administration of the free drug. (e, f) AZD-UPS nanodrug leads to potent tumor growth inhibition and extended survival of B16F10 melanoma bearing mice. The AZD-UPS therapy synergizes with PD-1 checkpoint blockade therapy. Modified and reprinted with permission from ref. [81]. Copyright 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

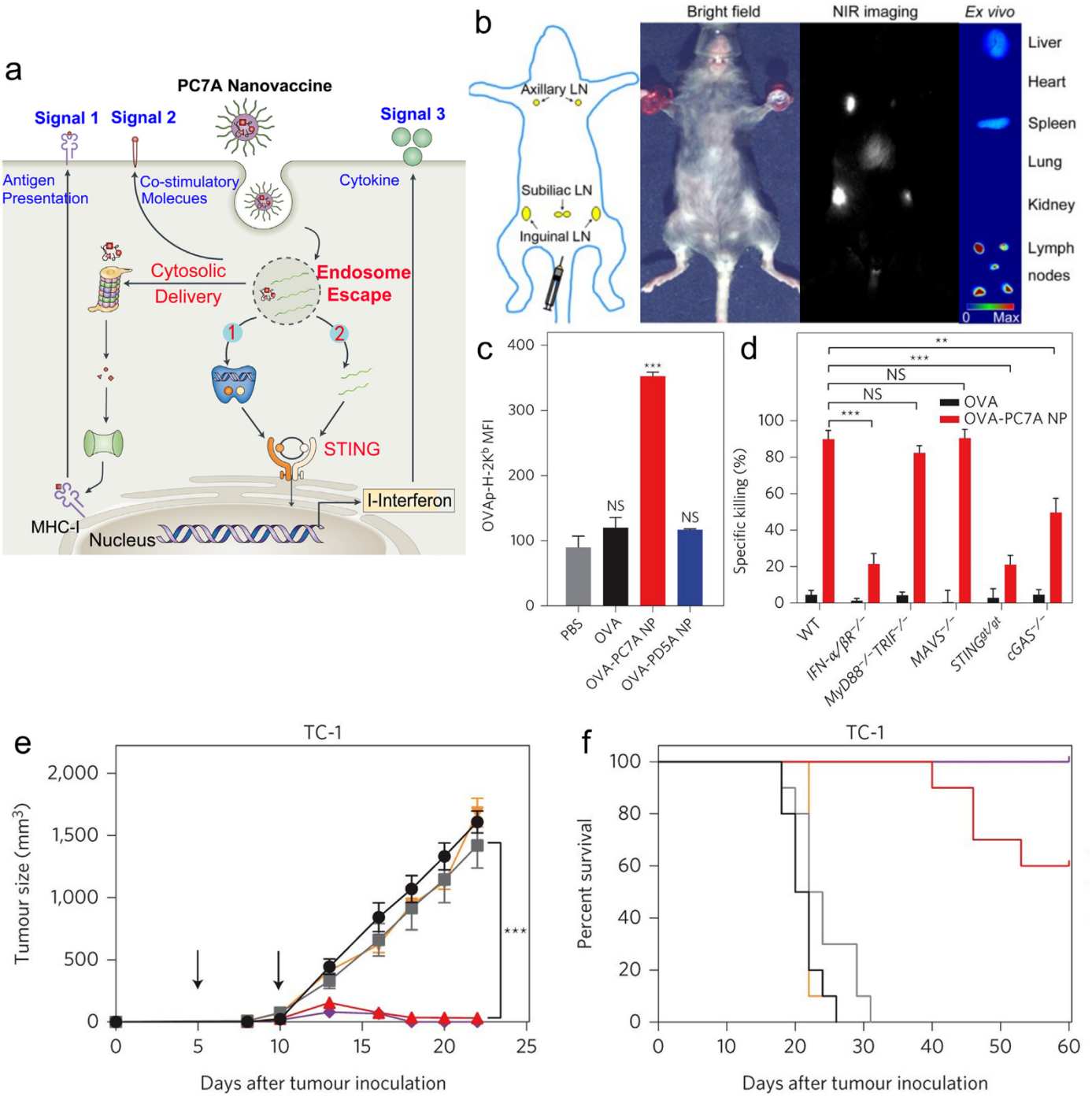

Fig. 6. PC7A nanoparticle vaccine induces robust T cell activation for cancer immunotherapy. (a) PC7A nanoparticle mediates antigen delivery and innate stimulation through the STING pathway for generation of cytotoxic T cells. (b) After subcutaneous injection, PC7A nanoparticles preferentially drain to lymph nodes. (c) Within lymph nodes, PC7A nanoparticles are endocytosed by antigen presenting cells for cytosolic delivery and cross presentation of the encapsulated ovalbumin cargo. (d) The generation of cytotoxic T cells by PC7A vaccination depends on the STING pathway. (e, f) Vaccination by PC7A induces robust anti-tumor immunity against HPV-induced TC-1 tumors, which synergizes with PD-1 checkpoint blockade therapy for complete cures. Modified and reprinted with permission from ref. [78]. Copyright 2017 Nature Publishing Group.

7. Conclusion and future outlook

Macromolecular cooperativity and phase separation are fundamental strategies biologic systems use to minimize entropy and maximize critical interactions between DNA, RNA, proteins and other biomacromolecules. Despite their biologic ubiquity, these processes have been absent from the medical armamentarium. Learning from nature, we propose to incorporate molecular and nanoscale cooperativity as a key design principle to improve drug efficacy, while minimizing off target toxicity frequently associated with the current systems.

In this review, we chose ultra-pH sensitive (UPS) nanoparticles to illustrate the implementation of cooperativity principle in drug delivery and therapy. UPS nanoparticles display an all-or-nothing assembly/disassembly phenotype responsive to environment acidification in solid cancers. In the example of AZD, UPS system escalated dose accumulation in solid tumors that synergizes with rapid drug release to achieve improved therapeutic outcomes. In cancer immunotherapy, we

discovered the polymer carriers can exert a pharmacologic effect through STING activation, enabling the design of multi-faceted vaccine therapy relying on fewer components.

The UPS nanoplatform illustrates the feasibility to allow precise control over the spatio-temporal orchestration of therapeutic signals. Looking into the future, the nanoparticle design could further incorporate positive and negative feedback mechanisms for precise control of pharmacological functions. Cholesterol delivery is regulated by positive feedback of endocytosis mediated by sequestered LDL receptors within clathrin-coated pits and negative feedback through SREBP-mediated downregulation of LDL receptors. While current drug delivery systems cannot incorporate such elaborate feedback mechanisms, design principles do exist for these loops based on carrier intrinsic properties. For example, UPS nanoparticles rely on positive feedback through micelle disassembly to increase dose accumulation and cooperative drug release. It is plausible that the encapsulated cargo could generate a negative feedback response. Metabolic acidosis in solid tumors is a result of rapid cell growth and altered energy metabolism of cancer cells. Delivery of an agent intended to slow the cancer cell growth or lead to cell death will undoubtedly slow the metabolic acidosis process, thereby decreasing the signal to which UPS nanoparticles respond. More directly, delivery of MCT inhibitors like AZD could exacerbate this effect. This self-limiting dosing strategy enlarges the therapeutic window of the therapy by allowing the administration of lower effective doses, limiting off-target toxicity, and improving tumor accumulation. We anticipate future nanomedicine will incorporate additional feedback controls to improve the precision of delivery and therapy.

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