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Convergence of nanomedicine and neutrophils for drug delivery

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ABSTRACT

Neutrophils have recently emerged as promising carriers for drug delivery due to their unique properties including rapid response toward inflammation, chemotaxis, and transmigration. When integrated with nanotechnology that has enormous advantages in improving treatment efficacy and reducing side effects, neutrophil-based nano-drug delivery systems have expanded the repertoire of nanoparticles employed in precise therapeutic interventions by either coating nanoparticles with their membranes, loading nanoparticles inside living cells, or engineering chimeric antigen receptor (CAR)-neutrophils. These neutrophil-inspired therapies have shown superior biocompatibility, targeting ability, and therapeutic robustness. In this review, we summarized the benefits of combining neutrophils and nanotechnologies, the design principles and underlying mechanisms, and various applications in disease treatments. The challenges and prospects for neutrophil-based drug delivery systems were also discussed.

1. Introduction

Nanotechnology has opened up new avenues for improving therapeutic outcomes and minimizing side effects, owing to its versatility, biocompatibility, and the property of controlled drug release. Nanoparticles offer numerous advantages over traditional small molecules in drug delivery, but they face significant challenges that hinder their therapeutic efficacy. One major challenge is their ability to penetrate biological endothelial barriers, such as vascular vessels and the blood-brain barrier. Moreover, limited penetration into the tumor microenvironment due to high tumor interstitial pressure poses an additional obstacle. These barriers and pressure restrict the entry of nanoparticles into target tissues and effectively infiltrate and distribute, limiting their effectiveness [1]. Additionally, rapid clearance rate and serum instability are also challenges for nanoparticles, resulting in suboptimal delivery efficiency [2]. Despite efforts to exploit the enhanced permeation and retention (EPR) effect for tumor targeting delivery, only a small percentage of nanoparticles successfully reach their target site [3–5]. Traditionally, polyethylene glycol (PEG) is commonly used to modify nanoparticles and provide stealth properties, reducing immune response and prolonging circulation [6]. However, some patients may develop antibodies against PEG, which can negatively impact the effectiveness and safety of the therapeutics [7]. Meanwhile, the exposure of PEG to the immune system can also induce severe inflammation due to the immunogenicity of the materials, even though low-molecular-weight PEG (200–400) could potentially reduce inflammatory cytokine production [8].

There are various materials and strategies in addition to PEG to provide nanoparticles with the stealth properties to evade immune recognition, including using endogenous cell membrane coating, and synthetic materials like zwitterionic polymers, polyglycerols (PGs) and so on [9,10]. Various types of cells, including red blood cells, platelets, macrophages, and cancer cells, have been employed in combination with nanoparticles for drug delivery [11–15]. Among these endogenous cell types, it is worth noting that neutrophils, the most abundant leukocytes in the human body, play an essential role as the first line of defense against invading pathogens [16]. Utilizing neutrophils for cell-based drug delivery possess immense potential as carriers for drug delivery, due to neutrophils' rapid response, tightly regulated and site-specific chemotaxis, and effective phagocytosis capabilities.

Neutrophil-mediated nano-drug delivery system leverages the unique capabilities of neutrophils to enhance nanoparticle functionality and improve drug delivery efficacy. Typically, one strategy is to utilize neutrophil membrane for nanoparticle coating and to create a cell-

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mimicking formulation. By coating with neutrophil membranes, the resulting hybrid structure exhibits improved biocompatibility, better tissue-targeting, and enhanced immune system-evading capability. Another approach directly utilizes neutrophils as carriers themselves. Neutrophils can actively internalize nanoparticles and transport them to the disease site during their natural recruitment process, serving as specialized delivery vehicles. This approach can be implemented in two ways: either by loading nanoparticles into isolated neutrophils prior to administration, or by in-situ hijacking neutrophils. Both approaches take advantage of the migratory and targeting capabilities of neutrophils, allowing for precise and targeted drug delivery. Complementary to the role of neutrophils, nanotechnology leverages the responsiveness of nanomaterials, such as pH or GSH-responsive disassembly capability, to further enhance targeting precision. Technologies such as ultrasound and irradiation further contribute to the precise manipulation of carriers, facilitating controlled drug delivery. Furthermore, genetic engineering techniques such as introducing CARs, can also be applied to neutrophils in combination with nanotechnology. Leveraging their unique properties and natural transport abilities, innovative neutrophil-based nano-delivery systems aim to enhance therapeutic outcomes and tackle challenges in various disease treatments, which opens up possibilities for more precisely controlled release of therapeutic agents, as well as the potential for diagnostic applications (Fig. 1).

The subsequent paragraphs will endeavor to provide a concise overview of the latest research developments, addressing the following key questions: (I) What are the distinctive characteristics and advantages of neutrophils that render them suitable for drug delivery? (II) How can these inherent properties be effectively incorporated into the rational design of a drug delivery system? (III) What are the current applications and challenges associated with the convergence of neutrophils and nanotechnology for targeted drug delivery?

2. Fundamentals for neutrophil-based nano-drug delivery systems

The utilization of neutrophil-based nano-drug delivery systems aim to address the fundamental challenge of achieving higher drug accumulation at the target site while minimizing the distribution of drugs in non-targeted organs and cells. The achievement of these delivery objectives relies on the unique properties of neutrophil-based nano-drug delivery systems. Apart from shared features for cell-based therapy, neutrophil-based nano-drug delivery systems hold significant promise in

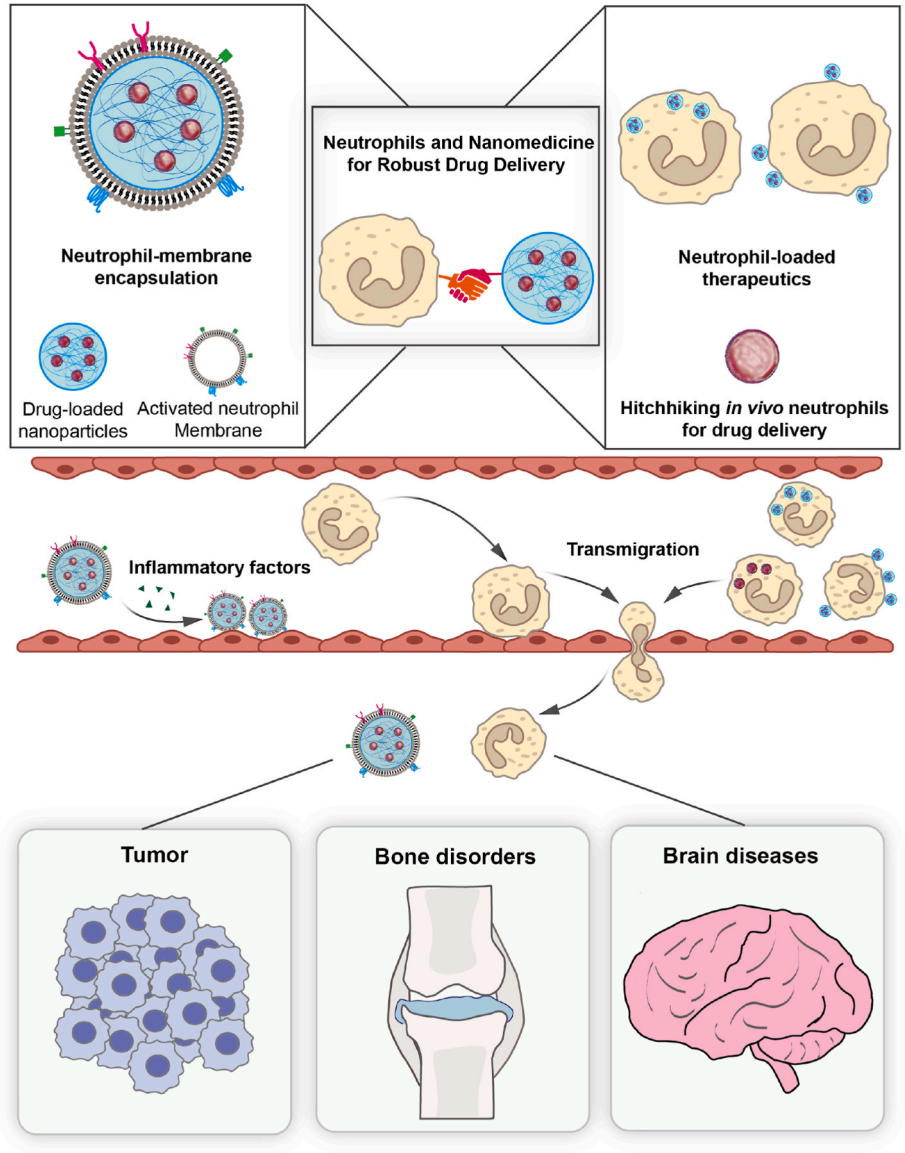

Fig. 1. Schematic illustration of the design principles of combining neutrophils and nanomedicine for applications in various diseases.

diseases associated with inflammation, offering a targeted and localized therapeutic approach. In addition, neutrophil-based nano-drug delivery systems demonstrate the potential in reaching sites that are typically inaccessible to traditional drug delivery methods due to biological barriers, such as blood-brain barrier.

The ability of neutrophils to undergo transmigration and chemotaxis plays crucial roles in their application as drug delivery vehicles. Neutrophils transmigrate across biological barriers through a process known as the neutrophil extravasation cascade. This cascade involves a series of well-coordinated steps including capturing, rolling, slow rolling, arrest, adhesion, crawling, and transmigration, enabling neutrophils to traverse from the bloodstream to the target site. Inactive and quiescent neutrophils will be rapidly activated and undergo an extravasation cascade beginning with the capturing of circulating neutrophils at the endothelial barrier surface. This initial step is facilitated by the interaction between upregulated adhesive molecules, such as selectins expressed on endothelial cells, and their corresponding receptors on neutrophils [17]. The weak and transient interaction enables dynamic and rolling motion through the lumina and allows neutrophils to search for signals that trigger activation. Upon exposure to the inflammatory cytokines secreted from disease sites, quiescent neutrophils rapidly become activated, and undergo firm attachment via integrins and morphology reshape [18]. Immediately after the adhesion, neutrophils will polarize to crawl on the endothelial surface in search of a suitable site for transmigration. Neutrophil crawling is facilitated by the involvement of specific integrins, including αLβ2 (also known as LFA-1), αMβ2 (also known as Mac-1), and α4β1 (also known as VLA-4), along with their ligands. The intercellular adhesion molecule 1 (ICAM-1), intercellular adhesion molecule 2 (ICAM-2), and vascular cell adhesion molecule-1 (VCAM-1) expressed on the endothelial cells also mediate tight adhesion, contributing to the crawling process [19]. Following crawling, neutrophils undergo polarization, wherein filamentous actins rearrange and form protrusions. This protrusion is important for neutrophils to sense and respond to the chemotactic gradients, allowing neutrophils to navigate through various layers, including the endothelial surface, pericyte layer, and venular basal membrane, to reach the underlying tissue. Despite this paracellular route, neutrophils can also directly cross through the endothelial cells, which is called transcellular diapedesis [20].

Physiologically, neutrophils are distributed in various organs and tissues, including the bone marrow, liver, lung, and spleen [21]. The bone marrow serves as a neutrophil reservoir for production and retention of mature neutrophils [22]. In normal conditions, after intravenous administration, neutrophils have a relatively short half-life of approximately 7 h in the vascular compartment. Subsequently, they leave the circulation and home to the bone marrow and liver for destruction and clearance [23]. In inflammatory conditions, neutrophils can target and migrate to the inflammatory sites owing to the mediation of chemotaxis. Chemokines orchestrate the entire process of neutrophil recruitment, from their release from the bone marrow to their directed migration. Various chemokines and cytokines act as mediators for controlling the selective recruitment of neutrophils, including colony-stimulating factors (CSFs), interleukin-8 (IL-8), tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and interleukin-1 (IL-1). This targeting ability of neutrophils, directed by chemokines, provides an opportunity to exploit their natural behavior for efficient drug delivery to diseased tissues [24,25].

3. Design principles of neutrophil-based nano-delivery systems

3.1. Neutrophil membrane-encapsulated nanoparticles

Coating nanoparticles with neutrophil membranes provides a shield that prevents the recognition and clearance by immune cells, thereby allowing nanoparticles to remain intact and biologically active for enhanced efficacy [26]. The transfer of surface markers, cell adhesion molecules, and receptors from neutrophil membrane to nanoparticles mediates the specific interaction with the target cells or tissues, facilitating targeted drug delivery.

3.1.1. Coating methods and mechanisms

Extrusion and ultrasonication are two typical methods for neutrophil membrane coating. The neutrophil membrane is first released using a hypotonic lysing buffer and homogenized using a tight-fitting pestle. In the ultrasonication method, the derived neutrophil membrane is incubated with nanoparticle cores and sonicated for surface wrapping (Fig. 2a) [27,28]. A shell-core structure is typically generated though this method. Another approach is extrusion, which offers the advantage of producing nanoparticles with relatively uniform sizes. The purified neutrophil membrane and nanoparticles are mixed and subjected to repeated extrusion through polycarbonate porous membranes with various pore diameters (Fig. 2b) [29]. Notably, when liposomes are utilized as synthetic cores for neutrophil membrane coating, the molar ratio of lipids to neutrophil membrane proteins becomes crucial in determining the nanoparticle characteristics such as size, polydispersity index, stability, and morphology. In this context, the neutrophil membrane can be either absorbed onto the surface of liposomes or embedded within the lipid layer of liposomes to form neutrophils-simulated liposomes when different ratios are applied. Even though the extrusion method is time-consuming, and the integrity of membrane coating and size distribution of particles are not optimal by ultrasonic method, the simplicity of these membrane coating methods enables versatile applications for a wide range of nanoparticle cores with different properties. Lipids (liposomes) [30], peptides, proteins, polymers [31,32], and inorganic materials like silicon dioxide [33] and gold are commonly used as nanoparticle cores in different contexts. Irrespective of the method employed for neutrophil membrane coating, a common challenge lies in achieving uniform coating integrity, often resulting in partial coverage of most nanoparticles [34]. This variability in coating integrity may lead to distinct uptake mechanisms, potentially compromising loading efficiency. Additionally, the substantial material loss during neutrophil membrane coating poses an obstacle for further scalability. While the ultrasonication method incurs less membrane material loss compared to the extrusion method, the coating efficiency of both methods remains an area for optimization. In response to these challenges, various novel membrane-coating technologies have emerged. Alternative methods, including electroporation and microfluidics, have also been developed to address the limitations associated with conventional methods [35,36].

Prior to membrane coating, lipopolysaccharide (LPS) can be employed as a stimulus to activate neutrophils. This activation induces an increased expression of adhesion molecules and chemotaxis receptors (Fig. 2b) [37,38]. These activated neutrophil surface proteins become instrumental for surface conjugation with nanoparticles or mediating nanoparticle uptake for drug loading. The disparity in surface proteins between quiescent and activated neutrophils also underscores the specificity inherent in *in-situ* neutrophil hijacking [39]. Activation not only enhances drug loading efficiency but also induces a higher targeting capability. These functions are activated in response to the initial stimulus, accentuating the critical role of activation in optimizing drug delivery outcomes. After membrane coating, the size of the particle slightly increases by approximately 10–20 nm. However, this size increase generally has a negligible impact on the delivery properties of the nano-formulation. Compared to other strategies like the anchoring of targeting peptides or antibodies, which require specific chemical groups for linkage or site modification, neutrophil membrane camouflage offers a broader range of applications.

3.2. Whole neutrophil-mediated drug delivery

Living neutrophils exhibit superior responsiveness to environmental changes, making them more precise and dynamic in delivering

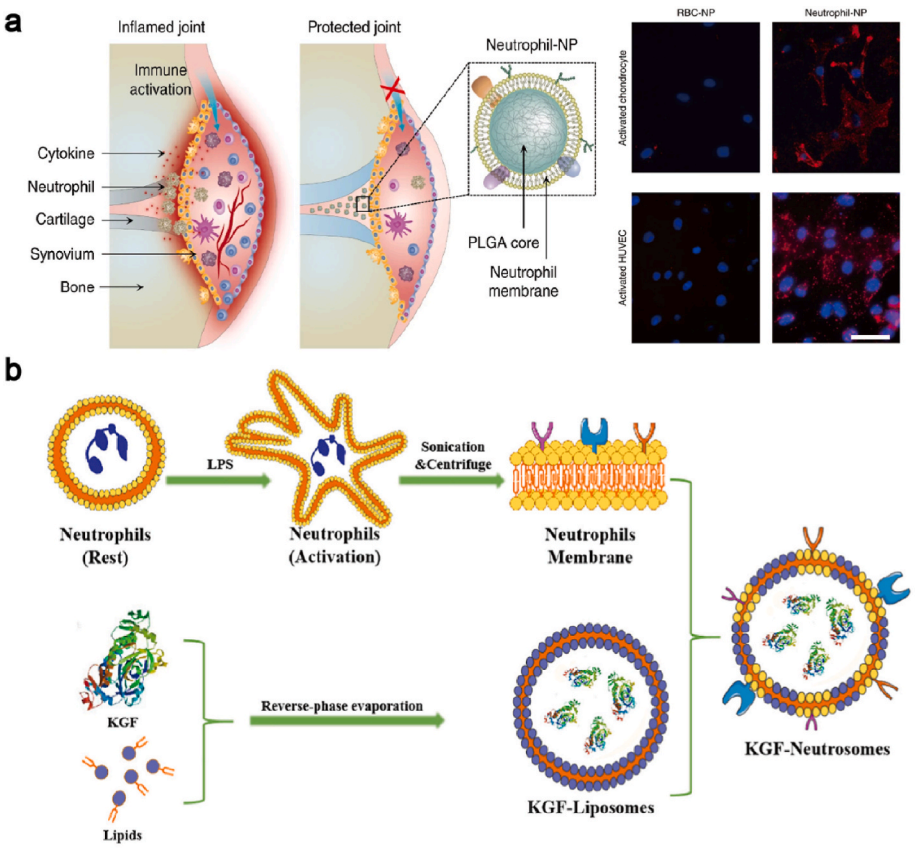

Fig. 2. Neutrophil membrane-coated nanoparticles. **a)** Schematic illustration of enhanced inflamed joint accumulation of neutrophil membrane coated Poly (lactic-co-glycolic acid) (PLGA) nanoparticles for synovial inflammation inhibition. Reproduced with permission [27]. Copyright 2018, Springer Nature. **b)** Schematic illustration of neutrophils activation, membrane isolation, and extrusion for generating neutrophils-simulated liposomes. Reproduced with permission [28]. Copyright 2019, Elsevier B.V.

therapeutic payloads. Unlike membrane encapsulation, the utilization of whole neutrophil preserves their intrinsic ability to respond to even subtle changes in the surrounding environment. The higher sensitivity to inflammatory signals increases the possibility of delivering therapeutics to specific types of cells. Furthermore, the large intracellular volume of living neutrophils enables them to accommodate a larger number of nanoparticles, achieving higher loading capacity and a higher drug-to-cell ratio. Additionally, multiple drugs can be loaded into neutrophils simultaneously, facilitating combinational therapy approaches.

There are typically two types of strategies, either generating drug-loaded neutrophils or hitchhiking in situ neutrophils for drug delivery. Nanoparticles are readily internalized by isolated neutrophils when incubating them in a suitable medium for approximately 1 h [40]. This method is relatively straight-forward, however, the short half-life of neutrophils, together with the potential risk of massive neutrophils extraction resulting in neutropenia, severe cytokine storm, and severe pulmonary complications with hypoxia and hypotension after infusion of a large number of neutrophils, still limits their extracorporeal loading and translation [41].

Without the process of isolating and culturing neutrophils, the strategies of *in-situ* hijacking neutrophils can remain the capabilities of chemotaxis and transmigration responding to the stimulus to the largest extent. When applying *in-situ* hijacking neutrophils for drug delivery, it is more critical to design functionalized nanoparticles for targeting activated neutrophils in the circulation or disease sites. Based on the upregulated membrane proteins and receptors on neutrophil surface after activation, as well as their physiological properties of neutrophils, nanoparticles are modified with corresponding targeting ligands to achieve high efficiency and specificity of in-situ hijacking. The rational choosing of targeting moiety and engineering nanoparticles to minimize off-target internalization by other cells will advance the application of in-situ hijacking neutrophils for targeted drug delivery for the treatment of cancer and other diseases.

3.2.1. *Versatile loading mechanisms*

The successful delivery of therapeutics by a neutrophil-mediated system is closely related to the loading capacity of the cells. Loading nanoparticles onto neutrophils can be achieved by including conjugating nanoparticles on the neutrophil surface, internalizing nanoparticles into neutrophils, and generating nanoparticle-containing neutrophil extracellular vesicles [42]. Importantly, the loading process does not significantly affect the functions of neutrophils, including cell migration, reactive oxygen species (ROS) generation, and inflammatory gene expression [43]. Once internalized, nanoparticles remain relative intact inside neutrophils for several hours before being released upon activation (Fig. 3a) [30]. However, it is of note that drug release in neutrophils even being protected by nanoparticles is still an issue that might dampens the functions and activities of neutrophils. Several internalization mechanisms can be applied, with the interaction between molecules on the neutrophils and particles playing a key role. Different patterns of molecules mediating nanoparticle internalization can be leveraged to provide versatile options to optimize drug delivery in various diseases, as listed below.

3.2.1.1. Receptor-mediated phagocytosis. Receptor-mediated phagocytosis can be utilized for loading drugs to neutrophils. These nanoparticles are specifically designed to engage Fcγ receptors (FcγRs) and can be efficiently recognized by activated neutrophils. During

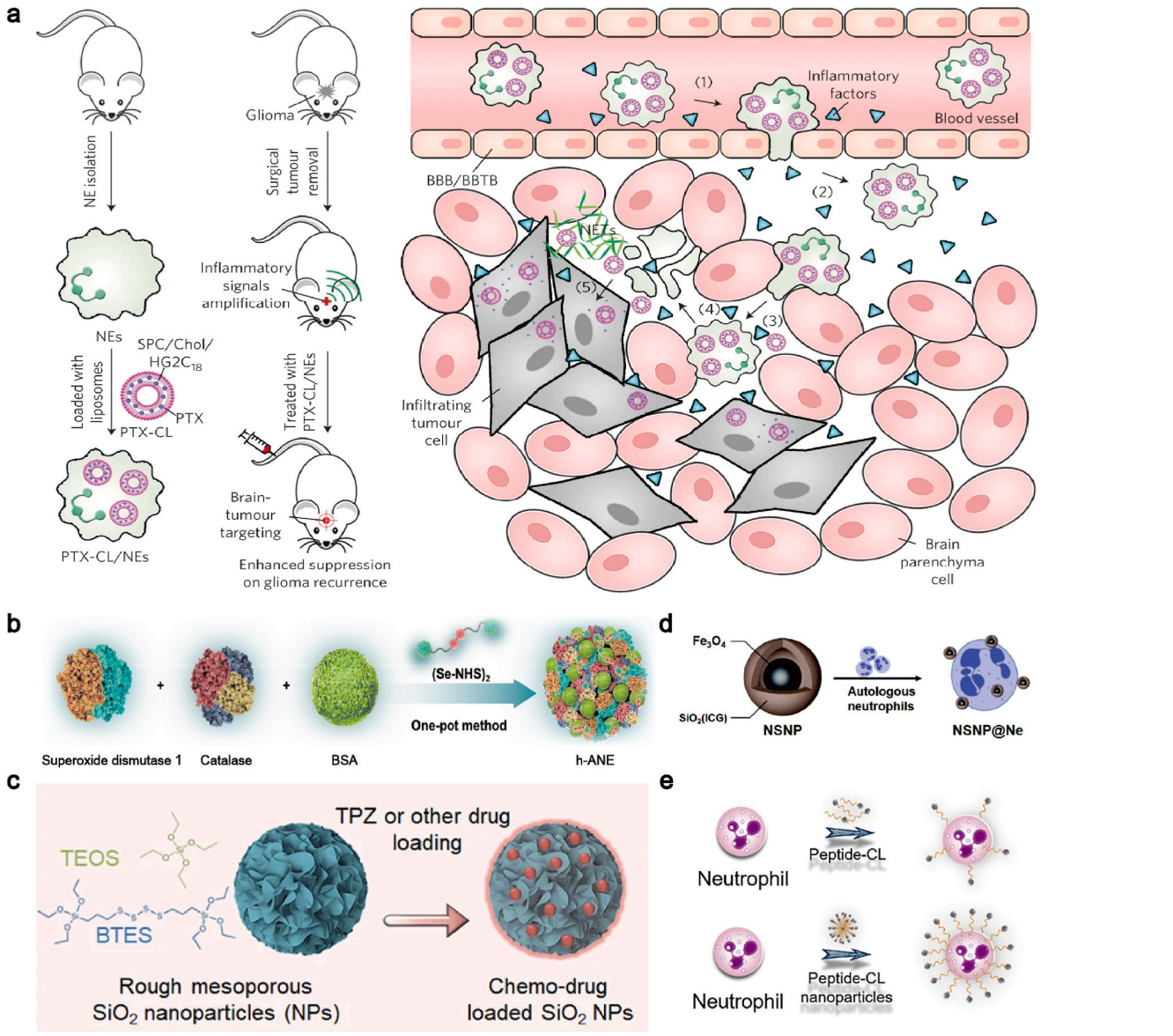

Fig. 3. Versatile mechanisms of loading nanoparticles into living neutrophils. **a)** The internalization of Paclitaxel (PTX)-loaded liposomes and inflammatory site-specific release for postoperative malignant glioma. Reproduced with permission [30]. Copyright 2017, Springer Nature. **b)** Receptor-mediated phagocytosis for loading into neutrophils. Reproduced with permission [44]. Copyright 2022, Royal Society of Chemistry. **c)** Rough mesoporous SiO_2 nanoparticles phagocytosed into neutrophils achieved 95 % loading efficiency. Reproduced with permission [45]. Copyright 2023, Springer Nature. **d)** Conjugation of nanoparticles on neutrophil surface through electrostatic attraction. Reproduced with permission [46]. Copyright 2021, Royal Society of Chemistry. **e)** Peptide-anchored nanoparticles on the membrane of neutrophils. Reproduced with permission [47]. Copyright 2023, Elsevier B.V.

inflammation, activated and adherent neutrophils exhibit higher expression of FcγRs, which are a major type of opsonin receptors (Fig. 3b) [44]. Denatured albumin-bound nanoparticles, generated through the desolvation of bovine serum albumin (BSA) and cross-linking of albumin, can be recognized by FcγRs of neutrophils for drug loading. *In vitro* studies have demonstrated the high uptake efficiency of these nanoparticles by neutrophils, with reaching up to 95 % of uptake. Moreover, the loading capacity of albumin-bound nanoparticles containing PTX can achieve 18 μg PTX per 1×10^6 neutrophils, highlighting their potential as effective drug carriers [48,49]. Researchers have developed a strategy to directly inject uniform and stable albumin-bound paclitaxel nanoparticles for antitumoral treatment [43]. Upon intravenous injection, the denatured albumin nanoparticles can be engulfed by activated and inflamed neutrophils through the Fcγ receptor III. Another study focused on the development of albumin nanoparticles for inflammatory lung diseases [39]. They further confirmed that the internalization process primarily occurs only in activated neutrophils induced by inflammatory factors such as TNF-α or LPS, rather than resting or circulating neutrophils. Approximately 30 % of the infiltrated neutrophils at the diseased site were observed to internalize the albumin nanoparticles within 20 h after injection. These results highlighted the effectiveness of *in-situ* internalization by activated neutrophils in the inflamed tissue via phagocytosis. However, it is important to consider the potential uptake of albumin nanoparticles by other cells expressing FcγRs, such as B cells, macrophages, and eosinophils, in order to assess the selectivity of FcγRs-mediated phagocytosis for albumin nanoparticles [50]. Besides albumin nanoparticles, PLGA was also studied for nanoparticle loading into neutrophil. The opsonization of PLGA nanoparticles in the blood circulation can lead to their phagocytosis by leukocytes for clearance, which significantly affects the desired drug delivery. However, this natural process of phagocytosis has inspired the specific uptake and delivery of PLGA nanoparticles by neutrophils.

Interestingly, a study has observed that neutrophils showed a preference for the uptake of PLGA nanoparticles of 260 nm diameters [51]. It is possible that the underlying loading mechanism is dependent on the antibodies or complement proteins recognized by phagocytic receptors [52].

In addition to opsonization-induced, receptor-mediated phagocytosis, other modification methods can be employed to enhance cell uptake of nanoparticles. One classical approach is the modification of ligands on nanoparticles. Integrin αvβ1, which is highly expressed on activated neutrophils, can be targeted to enhance the internalization of nanoparticles through receptor-mediated phagocytosis. A promising ligand for this purpose is the Cyclo (Arg-Gly-Asp-*d*-Tyr-Lys) (cRGD) peptide, which has a high affinity to neutrophils and monocytes by binding to integrin αvβ1 [53]. The dissociation constant (KD) values of the cRGD peptide to αvβ1 protein can reach 1.392×10^{-7} M. When liposomes are modified with cRGD, neuroprotective agents loaded in cRGD-anchored liposomes can selectively accumulate in the impaired parenchyma regions, where traditional drug delivery methods are hindered by insufficient blood supply. The uptake of cRGD-liposomes can be significantly increased, at approximately 8-fold. Though the uptake by monocytes and neutrophils may be at a similar level, the large number of neutrophils among white blood cells makes their contribution critical [54]. Similarly. Cationic liposomes modified with anti-CD11b antibodies have been used for neutrophil-mediated delivery to treat postoperative glioma recurrence, inflamed skeletal muscle, and ischemic heart [43,55]. CD11b, also known as integrin αM or Mac-1, is highly expressed on activated neutrophils [56]. Gold nanorods linked with anti-CD11b antibodies can be internalized and actively delivered to tumor sites, with a size range of 200–250 nm. In an acutely inflamed tumor model, up to 50 % of neutrophils could successfully internalize CD11b antibody-decorated nanoparticles [57]. It is important to note that CD11b, as a neutrophil surface protein, also play a crucial role in regulating the adhesion and migration of neutrophil. While the use of anti-CD11b antibodies can enhance nanoparticle uptake, there is also a risk of blocking this antibody might sacrifice neutrophil chemotaxis [43]. Other upregulated receptors such as C-X-C motif chemokine receptor type 2 (CXCR2) can be involved in nanoparticle targeting neutrophils and loading. The tripeptide agonist N-acetyl Pro-Gly-Pro (Ac-PGP) with a high affinity to the CXCR2 receptor has been developed for delivering macromolecular protein-based drugs to cerebral ischemia area [58].

Neutrophils express various surface receptors for recognizing pathogens through pathogen-associated molecular patterns (PAMPs). LPS, Cytosine-phosphorothioate-guanine (CpG), and many other bacterial and viral components can be efficiently captured by Toll-like receptors [59]. Those receptors capable of recognizing microbial structures enables efficient phagocytosis and uptake of pathogen membrane-coated nanoparticles. The membrane of *Escherichia coli* have facilitated drug delivery by hijacking neutrophils while preventing premature drug release within neutrophil carriers [60]. To alleviate the difficulty of productivity and cumbersome extraction by using natural bacterial membranes, mycoplasma membrane has also been utilized as an alternative approach [61]. Overall, leveraging the receptor-ligand interaction for phagocytosis not only enhances the specificity for neutrophils compared to other immune cells based on the different surface receptor expressions after activation but also improves the internalization efficiency.

3.2.1.2. Size, shape, and surface roughness-preferred phagocytosis. Neutrophils preferentially phagocytose particles with specific physical particle parameters including the size, shape, and surface roughness. Particle size impacts the interaction with neutrophil membrane, leading to different uptake efficiency by neutrophils with the preference for larger particles (100–200 nm) [62]. It has also been reported that neutrophils have increased phagocytosis towards microbial pathogens with rough surfaces or rod-shaped particles, distinguishing neutrophils from macrophages and other mononuclear phagocytes [63,64]. The number of rods internalized by neutrophils could achieve around 3.5-fold higher than spheres, which is a characteristic that is unique to neutrophils. The underlying mechanism might be increased surface motility of neutrophils and the phagocytosis independent on phosphorylation of FcγR. As for the loading, this property can be exploited by rationally designed nanoparticles with desired physical properties, maximizing drug loading and specifically targeting neutrophils among various leukocyte subpopulations. Chang et al. used rough mesoporous SiO_2 nanoparticles for chemo-drug loading, with up to 12 % nanoparticles being loaded in 95 % neutrophils (Fig. 3c) [45]. This study has also demonstrated higher uptake of rough SiO_2 nanoparticles compared to smooth SiO_2 nanoparticles by neutrophils.

3.2.1.3. Clathrin-mediated endocytosis and micropinocytosis. Clathrin-mediated endocytosis is a process that transports various molecules from the cell surface into the intracellular cytoplasm, during which the clathrin binds to the transmembrane or extracellular molecules to enrich them to the region that will form vesicles and undergo endocytosis [65]. The initiation of clathrin-mediated endocytosis might occur randomly, and certain lipids or proteins possess properties that promote endocytosis [66]. While the specific role of clathrin-mediated endocytosis in neutrophil loading is not fully understood, several studies have at least demonstrated its involvement in the loading process. For instance, when neutrophils were treated with inhibitors of micropinocytosis or clathrin-dependent endocytosis, such as colchicine or oxophenylarsine (PhAsO), the internalization of nanoparticles generated from (Pro--Gly-Pro tripeptide)-PEG-(Dendrigraft poly-L-lysine)-(N-succinimidyl 3-(2-pyridyldithio)-propionate) (PGP-PEG-DGL-PDP) polymer with size around 100 nm was significantly inhibited [58].

3.2.1.4. Surface attachment. Surface conjugation provides an alternative approach for delivering drug-loaded nanoparticles by directly attaching them to the surface of neutrophils. Neutrophils have a positive surface potential, and negatively charged nanomaterials can be attached to neutrophil surfaces through electrostatic attraction [67]. Wang et al. have generated magnetic silica particles with a negative surface potential of -37.5 mV and attached them to the surface of neutrophils (Fig. 3d) [46].

While this loading mechanism might elicit minimum impact on the morphology of neutrophils, this type of attachment could lead to undesired payload release during transportation due to relatively weak interaction. To achieve more stable conjugation, click reaction and the binding between ligands and receptors can also be applied. For instance, Hao et al. employed hyaluronic acid terminated by a maleimide group to generate hyaluronidase-responsive nanoparticles containing stimulators of interferon genes (STING) agonists. These nanoparticles were further linked to the converted thiol group on the neutrophils' surface via click reaction [68]. The vasculature extravasation and tumor penetration of neutrophils promoted the penetration and tissue accumulation of STING in triple-negative breast cancer (TNBC). The STING agonists stimulated an innate immune response, directing neutrophils toward a tumoricidal state and introducing a tumor-killing effect by ROS release. Another approach involves utilizing the pair of the receptor and ligand without the internalization effect for surface conjugation. For example, the cinnamoyl-F-(D)L-F-(D)L-F (cFLFLF) peptide can bind to the N-formyl peptide receptor 2 (FPR2) receptor which is overexpressed on the neutrophil membranes. This peptide-CL can be effectively anchored onto the intrinsic circulated neutrophils for drug loading (Fig. 3e) [47].

3.2.2. Payload release mechanism

Preventing the payload release during transportation and ensuring controlled release at the desired site are critical for effective neutrophil-based nano-drug delivery systems. It is ideal for the payloads to remain

intact within neutrophils before reaching the target sites without affecting the normal physiology and function of neutrophils. At the same time, premature drug release before neutrophils reaching the target sites can result in potential side effects due to drug accumulation in the circulation system and other tissues. Meanwhile, it is also of great significance for neutrophils to release an adequate number of payloads at the desired sites in order to achieve therapeutically effective drug concentrations. There are several strategies for increasing the stability of nanodrugs internalized by neutrophils. Materials like glutathione-responsive mesoporous organic silica nanoparticles demonstrated good stability during neutrophil migration and in vitro coculturing with cancer cells [45]. The inducible release profile provided by stimuli-responsive nanomaterials protect drug in nanoparticles from uncontrolled release. Others have also used the *E. coli* membrane to improve the biocompatibility of nanoparticles within neutrophils and limited drug release inside neutrophils for at least 6 h [60].

While a more comprehensive understanding is required to elucidate the precise mechanisms of payload release from neutrophils at the intended time and sites, it is generally believed that the formation of neutrophils extracellular traps (NETs) and the action of degranulation play important roles in many delivery strategies. NET, which is a part of the immune system, is an extracellular structure composed of DNA, histones, and granular proteins [69]. NETosis is the process of neutrophils releasing NET, which is responsive to the pro-inflammatory stimuli, like TNF and IL-8, and promoted by a higher level of ROS [70]. This presents an opportunity for simultaneous nanoparticle release from the cytoplasm of neutrophils [71]. Of note, although neutrophils can generate exosomes which are small vesicles that are associated with intercellular communication, they are not involved in payload release [72]. Notably, it is possible that the rapid apoptosis of neutrophils induced by the sustained release of NETs under continuous inflammatory signals can be beneficial in protecting the neutrophils from the payload contained within cells. In addition to NETosis, neutrophils can also release their granular contents by degranulation. When the granules translocate to dock and fuse with the cell membrane, the encapsulated nanoparticles present within the cells can also fuse and be released extracellularly [73]. Furthermore, the induced phagocytosis of apoptotic neutrophils can be helpful as a way for payload delivery. For example, researchers have used neutrophils to deliver siTNFα-loaded liposomes for rheumatoid arthritis. Both neutrophils and macrophages are actively involved in the high level of TNFα in the inflammatory network. After incubating siTNFα liposomes with neutrophils, the drug-loaded neutrophils after administration are recruited to and accumulated at the inflammatory sites. The liposomes underwent lysosome escape and the siTNFα within neutrophils repressed the translation of TNF-α. Additionally, siTNFα can also be transferred to macrophages in the rheumatoid arthritis by active phagocytosis of apoptotic neutrophils containing siTNFα by macrophages, as well as through the uptake of siTNFα liposomes after NETs formation for drug release [74]. Of note, several studies also observed a contact-directed transport, an endocytosis-independent transport of the payload from neutrophils to the target cell, especially to the neutrocytes [54,58]. Through proximity, the formation of bridges, membrane transferring, and deformation of neutrophils and exosomes have all participated in the payload transportation. However, the detailed mechanism and its difference from other release methods have not been elucidated yet.

Despite the natural process of neutrophils for payload release, researchers have demonstrated a method to trigger payload release from neutrophils using near-infrared irradiation [30]. Under 808 nm laser irradiation, neutrophils will be destroyed which leads to their fragmentation into debris, and the payload will be successfully released. The accumulated release can be significantly increased from approximately 5 % to 80 % within 2 h. This manipulation of drug release allows for localized therapy and provides a controlled manner for the delivery of therapeutics.

4. Applications of neutrophil-based nano-drug delivery systems for targeted and programmable therapy

4.1. Targeted therapy

Utilizing the unique tropisms and recruitment capability of neutrophils, researchers can target drug delivery to specific sites within the body. Neutrophil targeting is based on the interactions between various molecules present on the surfaces of neutrophil membranes with other cells or with the biological environment. The cocktail of abundant and diverse proteins in neutrophils plays an essential role in the targeting process. Additionally, various adhesion molecules and cytokines are also employed in targeting certain tissues or disease sites using neutrophil-based nano-drug delivery systems. Theoretically, depending on the characteristics of diseases and the involvement of inflammatory signals, neutrophils can be used for targeting various destinations in different diseases through chemotaxis. This targeting approach holds great promise for numerous diseases and applications. Below are several representative examples of applying neutrophil-based nano-drug delivery systems for targeted therapy.

4.1.1. Brain disorders

The transmigration of neutrophils across endothelial barriers is particularly beneficial in delivering therapeutics to treat diseases in the brain that are typically inaccessible due to the existence of blood-brain barrier (BBB) [75]. It is also commonly believed that an inflammatory condition is shared by many brain diseases, such as cerebral ischemia, glioma, and other neurological diseases [76]. Back in 2003, there were studies using magnetic liposomes for monocytes/neutrophil-mediated brain targeting, which opened a new perspective for the treatment of brain disorders [53].

Cerebral ischemia occurs when there is insufficient blood flow to the brain, resulting in brain damage. Neutrophils have been found to play a significant role in ischemia stroke, with increased infiltration of circulating neutrophils in the acute phase and subsequently amplified recruitment of infiltrated leukocytes in the ischemic area [77]. The recruitment of neutrophils to the ischemic area typically begins within 30 min and can continue for over 15 days post-ischemia. This prolonged recruitment period highlights the potential of neutrophils in targeted drug delivery [78]. The rapid and sustained response of neutrophils to the ischemic area, as well as their transmigration and chemotaxis abilities, makes them a promising candidate for the treatment of ischemia. Several strategies utilizing neutrophil-biomimetic or neutrophil-mediated approaches have been developed to treat ischemic brain damage. The principle behind these strategies involves several steps. First, neutrophils carrying therapeutic drugs transmigrate across the BBB. Next, owing to the chemotactic abilities, neutrophils specifically penetrate and accumulate in the ischemic brain region (Fig. 4a). Finally, the loaded drug can be effectively released under specific stimuli, exerting pharmacological effects on neurocytes or remodeling disease microenvironment [58]. One approach utilizes liposomes loaded with neuroprotective agents and modified with cRGD peptides. Due to the high affinity of cRGD to integrin αvβ1, these liposomes can be efficiently internalized by blood neutrophils, which then deliver drugs to the ischemic region of the brain. This strategy takes advantage of the secondary inflammation that occurs after ischemia, extending the time window for neuroprotective treatment (Fig. 4b) [54]. In addition, the use of cationic liposomes with a positive surface charge enhances the interaction between liposomes and neutrophils, promoting higher drug loading efficiency in neutrophils for ischemia treatment [79]. Employing neutrophils as carriers enables the specific delivery of various neuroprotective medications to the ischemic area. Simultaneously, neutrophil-biomimetic nanomedicine acts as a nano-buffer to neutralize detrimental agents and prevent neuronal erosion in the penumbra [38]. This nanomedicine is formed by coating neuroprotective-loaded nanoparticles with neutrophil membranes, and

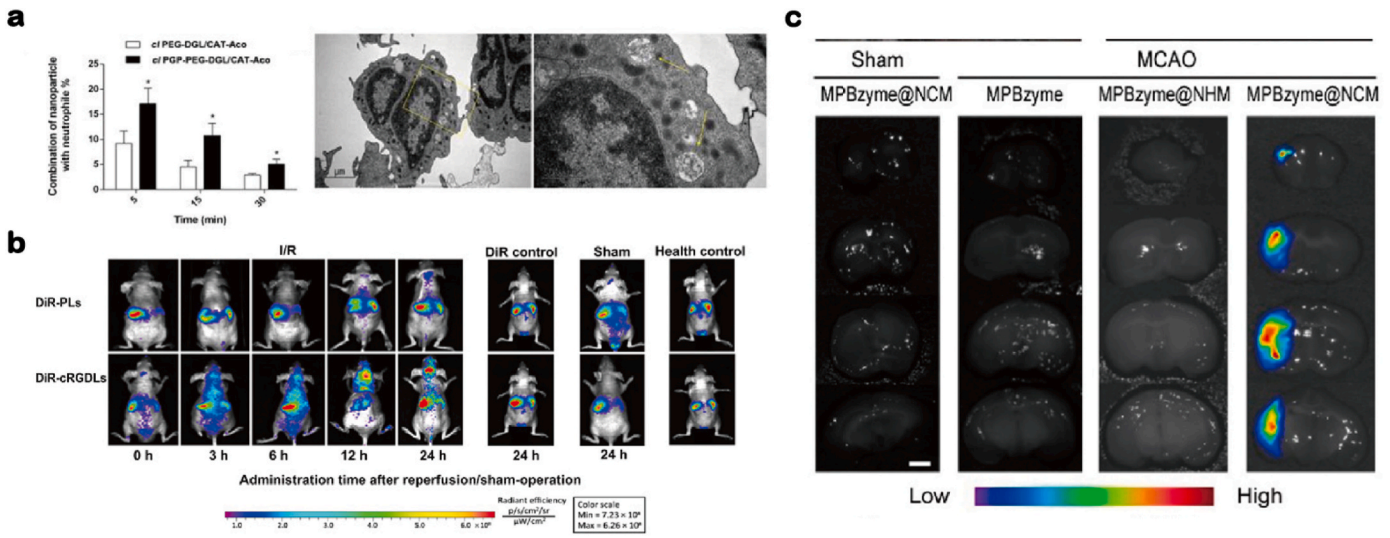

Fig. 4. Taking advantages of neutrophils to cross the blood-brain barrier for brain diseases. **a)** The extent of nanoparticles endocytosed within neutrophils over time after injection. Reproduced with permission [58]. Copyright 2017, Ivyspring International Publisher. **b)** Fluorescence imaging of mice after delivering by neutrophils to the ischemic brain. Reproduced with permission [54]. Copyright 2019, American Association for the Advancement of Science. **c)** Images of brain coronal sections in sham-operated mice and stroke mice indicating inflammation recruited neutrophil membrane-coating nano-enzyme. Reproduced with permission [80]. Copyright 2021, American Chemical Society.

an antioxidant is introduced onto the particle to generate a corona. The corona rapidly eliminates ROS, contributing to the remodeling of the ischemic microenvironment. More importantly, the neutrophil membrane used for transportation across the BBB and the recruitment can absorb inflammatory cytokine, including interleukin-1 beta (IL-1β) and TNF-α through receptor binding. This process plays an important role in detrimental agent buffering for ameliorating inflammatory conditions in the ischemic brain. In another study, nano-enzyme coated with neutrophil membrane showed excellent targeting capabilities, with significantly higher accumulation observed in the ipsilateral brain area compared to the blood and contralateral brain area [80]. These neutrophil-like nanozymes specifically target microglia after penetrating, rather than NeuN^{+} cells or GFAP^{+} cells. Therefore, a more precise targeting approach holds promise for delivering therapeutic

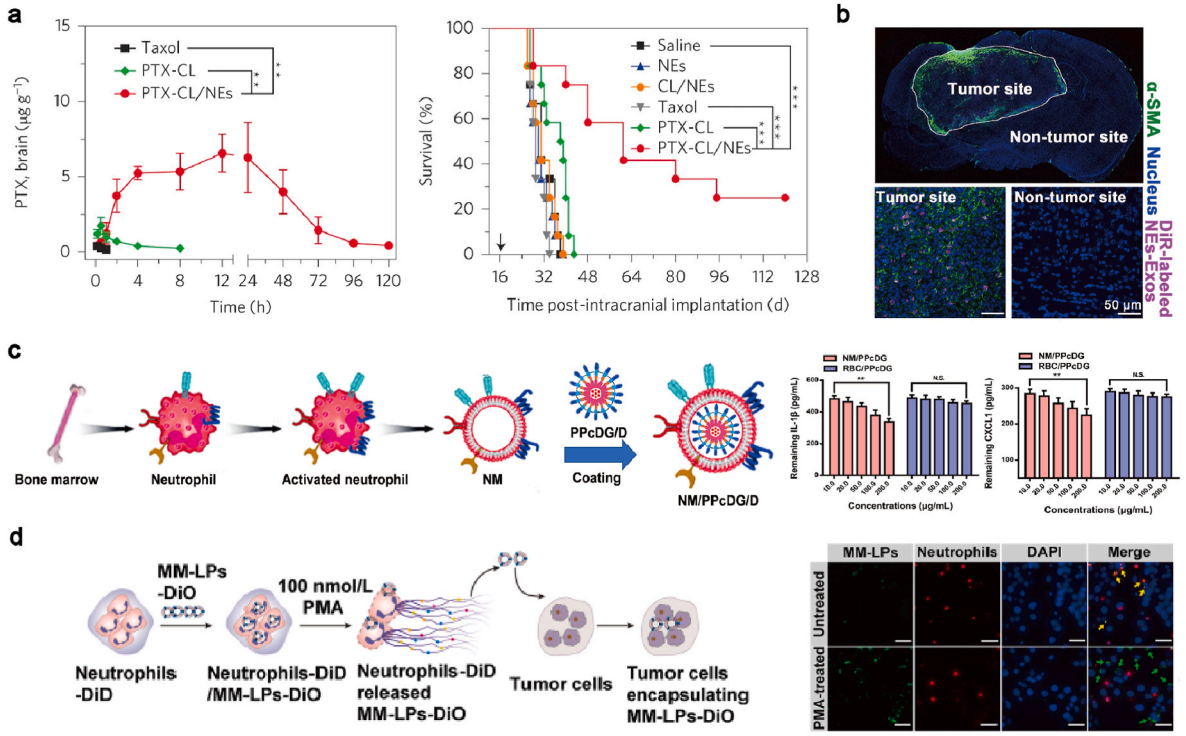

Fig. 5. Applications of combining neutrophils and nanoparticles for cancer treatment. **a)** Treatment efficacy of PTX-loaded liposomes delivered by neutrophils for glioma. Reproduced with permission [30]. Copyright 2017, Springer Nature. **b)** Superior glioma tumor-targeting ability of DiR-labeled neutrophil exosomes in vivo. Reproduced with permission [42]. Copyright 2021, Elsevier Ltd. **c)** Neutrophil membrane serves as sponge to absorb cytokines for controlling malignant tumors. Reproduced with permission [87]. Copyright 2023, Acta Materialia Inc. **d)** The apoptosis and NETs of neutrophils induced by inflammatory signals in the tumor microenvironment for loaded-contents release. Reproduced with permission [61]. Copyright 2022, Elsevier B.V.

agents to certain cell types within the brain for the treatment of ischemia (Fig. 4c).

Glioma, one of the most aggressive tumors of the central nervous system (CNS), presents significant challenges for drug delivery, impeded by its location and the presence of the BBB. Additionally, the persistent inflammation in the tumor microenvironment further complicates therapeutic interventions [81]. Neutrophils-based drug delivery platforms have shown promise in overcoming these challenges by proactively penetrating the BBB even for the intact BBB, and efficiently delivering therapeutic drugs to glioma tumor tissue. For example, in postoperative glioma models, neutrophils can be utilized to carry chemotherapeutic drugs such as PTX loaded in cationic liposomes by intravenous injection. Superior targeting ability of brain tumors and increased accumulation of PTX level enabled robust cytotoxicity of cancer cells (Fig. 5a) [30]. When applying neutrophil-mediated delivery strategies to post-surgical glioma mice models, the neutrophil recruitment for targeted delivery replies on not only the pro-inflammatory cytokines such as TNF-α, Interleukin 10 (IL-10), and Interleukin 6 (IL-6) in the glioma tumor microenvironment, but also the released inflammatory signals after tumor resection [72]. While the effectiveness of a neutrophil-based delivery strategy in the orthotopic glioma tumor without surgery might be suboptimal compared to post-surgery tumors, it is still effective with additional efforts to enhance the targeting ability (Fig. 5b). For example, Li et al. employed neutrophils as BBB carriers for the delivery of a hollow sono-sensitive TiO_2 shell trapped with PTX-loaded liposome. Upon the ultrasound irradiation in the glioblastoma (GBM), the generated ROS by the TiO_2 shell destroyed the structure of the liposome, resulting in the site-specific anti-PD1 antibody release. The resulting tumor-killing effect and induced inflammation are beneficial in amplifying neutrophil recruitment for targeted drug delivery. A precisely controlled drug release has been achieved to minimize the side effects of the chemotherapeutics [82]. Given that macrophages are also actively involved in GBM, integrating diverse functionalities from both macrophages and neutrophils can exert outstanding targeting properties. It has been shown that chemotactic stimuli and attractants secreted by GBM stem cells are attributed to the recruiting process, drawing on each other's merits for better delivery (Fig. 6a) [83]. Furthermore, considering that tumor-associated neutrophils demonstrate both pro-and anti-tumor functions in the GBM tumor microenvironment, neutrophil exosomes containing anti-cancer drugs are applied to prevent any potential pro-tumor effect from neutrophils [42,84].

4.1.2. Cancer

Oncogenic changes in tumors often lead to the development of an inflammatory tumor microenvironment, characterized by the presence of inflammatory cells and molecules, including chemokines and cytokines. In various cancer, elevated levels of chemokines such as TNF-a, IL-1β, IL-6, IL-23, IL-8, chemokine (C–C motif) ligand 2 (CCL2), and chemokine (C–C motif) ligand 20 (CCL20) have been observed [85]. These chemokines and inflammatory tumor microenvironment create a favorable environment for neutrophil infiltration and accumulation at tumor sites. This phenomenon also demonstrates the potential for neutrophil-based nano-drug delivery systems to deliver therapeutics to the tumor microenvironment. Meanwhile, activated neutrophils exhibit upregulation of specific proteins and adhesion molecules on their surface, such as L-selectin, lymphocyte function-associated antigen 1 (LFA-1), β1 integrin, C-X-C chemokine receptor type 4 (CXCR4), macrophage-1 antigen (MAC-1), platelet endothelial cell adhesion molecule-1 (PECAM-1), and P-selectin glycoprotein ligand-1 (PSGL-1). These molecules have a high affinity for receptors expressed on certain types of cancer cells, making solid tumor tissues and specific tumor cells targetable by neutrophil-based nano-drug delivery systems [86]. The use of neutrophil-based nano-drug delivery systems extend beyond therapeutic delivery, as they have been employed to absorb the cytokines present in the tumor tissue. Given that these cytokines are essential for the recruitment of myeloid-derived suppressor cells, which is beneficial to metastasis, ameliorating the microenvironment in a post-operative inflammatory site, can effectively prevent the formation of a pre-metastatic niche (Fig. 5c) [87].

In the context of lung cancer, chemotherapeutic has been delivered by peripheral blood neutrophils in an on-demand manner. Neutrophils

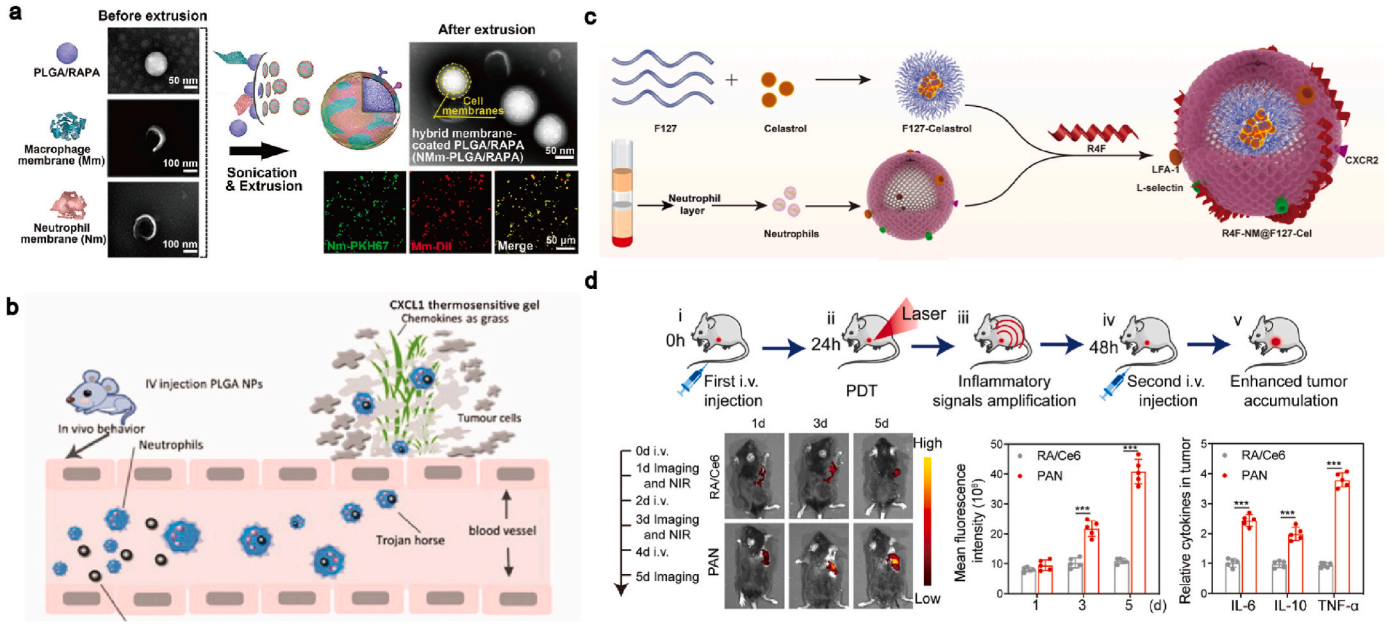

Fig. 6. Enhancing targeting capability for tumor therapy. **a)** Dual neutrophil-macrophage membrane coating of nanoparticles for complementary targeting. Reproduced with permission [83]. Copyright 2021, Elsevier B.V. **b)** Using CXCL1 chemokine-laden polymer hydrogels for attracting neutrophils. Reproduced with permission [51]. Copyright 2019. **c)** Targeting macrophages in rheumatoid arthritis by peptide anchoring and neutrophil membrane coating. Reproduced with permission [102]. Copyright 2023, Springer Nature. **d)** Inflammatory signals induced by photodynamic therapy for amplification and accumulation of photoactive neutrophil in tumor. Reproduced with permission [93]. Copyright 2021, Elsevier Ltd.

expressing a high level of sialic acid-binding Ig-like lectin-F (SiglecF) were found to preferentially accumulate within lung tumor sites [88]. Utilizing the binding receptors 1-selectin or CD62L on peripheral blood neutrophils, poly (sialic acid) decorated liposomes were targeted to these peripheral blood neutrophils, leading to enhanced uptake via receptor-mediated phagocytosis. The pixantrone-loaded liposomes were efficiently delivered to the lung cancer sites due to the pronounced neutrophil accumulation in the lung tumor [89]. Additionally, circulating neutrophils can be attracted to tumor sites by using a dual Toll-like receptor 7/8 (TLR7/8) agonist R848, which induced a potent immune response and significantly increased the proportion of neutrophils in the tumor, potentially enhancing targeting during immune response in a 4T1 breast cancer model (Fig. 5d) [61].

Moreover, recent advances have shown promise in targeting traditional difficult-to-treat cancers, such as triple-negative breast cancer and pancreatic cancer, based on the interaction of activated neutrophils with the receptors specifically expressed on those cancer cells [90]. Neutrophil membrane-coated nanoparticles can precisely target triple-negative breast cancer cells while sparing normal breast epithelial cells. Therefore, this approach restricts the anti-proliferative and cytotoxic effect of delivered therapeutics to cancer cells, minimizing toxicity to other normal cells. Similarly, naïve neutrophil membrane coating can confer drug-loaded nanoparticles with chemotaxis properties and enhanced uptake by pancreatic cancer cells [91].

In addition to leveraging the natural inflammation signals in cancer progression, researchers have explored the introduction of other molecules to amplify these signals, thereby enhancing the targeting of neutrophil-based nano-drug delivery systems. One such approach involves implanting CXCL1 chemokine-laden polymer hydrogels at the surgical tumor sites (Fig. 6b). The CXCL1 is known to play a crucial role in attracting neutrophils to the inflammatory sites. By incorporating CXCL1 into hydrogels, a controlled and continuous release of inflammatory chemokines and signals stimulates the recruitment of neutrophils mimicking PTX-loaded nanoparticles [51]. Antibodies can also be applied to enhance the targeting ability of neutrophils to cancer cells. Researchers have utilized TA99, an antibody against gp75 antigens that are found specifically in melanoma. The recruitment of neutrophils to the tumor sites is dependent on the antibody-dependent cell-mediated cytotoxicity (ADCC) effect. When TA99 antibodies bind to the gp75 on melanoma cells, the Fc domain of the antibody is exposed. Neutrophils, which express Fc receptors will recognize the bind at the Fc region anchored to the tumor cells. This interaction increases the accumulation of nanoparticle-laden neutrophils at the tumor site, with increments from 0.7 % to 6.2 % [57]. To further improve anti-tumor efficiency, enhancing the interaction between nanoparticles and cancer cells through surface modification is a promising strategy. For instance, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been decorated to the surface after neutrophil membrane coating [92]. TRAIL is a ligand that binds to the death receptors 4 and 5 (DR4 and DR5) on tumor cells, initiating mitochondrial-dependent cell death and triggering apoptosis. The recognition of tumor cells by TRAIL enhanced the uptake of chemotherapeutic drugs in nanoparticles and induced cancer cell death. Neutrophil membrane camouflaging on TRAIL-decorated nanoparticles significantly prolonged the blood circulation duration of TRAIL, which ensured that TRAIL could elicit its cytotoxicity before being cleared from the bloodstream. Neutrophil-based nano-drug delivery systems can also serve as a platform for intracellular drug delivery. By combining the neutrophil membrane-coated nanoparticles with other targeting moieties specific to subcellular sites, a highly targeted delivery of therapeutics can be achieved. For example, a mitochondrion-disrupting peptide can be internalized and accumulate in the mitochondria after being encapsulated in the neutrophil membrane-coated nanoparticles [93].

Photosensitization and photothermal therapy are the latest techniques that convert electromagnetic radiation into other energy forms for therapeutic purposes. In photosensitization, electromagnetic radiation generates a range of ROS, while in photothermal therapy, it directly converts into heat energy to kill cancer cells. Both therapies can induce acute and localized inflammation within the tumor site, which leads to the rapid activation and infiltration of neutrophils, enabling neutrophil-mediated drug delivery [32]. For cancer treatment, current photothermal therapy possesses the restriction for inadequate accumulation of photothermal agents for effective killing outcomes and a high risk of tumor recurrence due to insufficient exposure in the tumor margin or deep regions. Also, the poor distribution in vivo after systemic administration might lead to undesired side effects such as hyperthermia. Several neutrophil-mediated strategies successfully improved the efficacy of nanoparticle-based photothermal therapy with the enhanced killing effect of photothermal therapy and significantly prevented recurrence [46]. The targeting and penetrating of neutrophils in the disease sites and camouflaging effect for reduced systemic clearance significantly increase the concentration of phototherapeutic agents in the desired locations. The internalization of those nanoparticles containing phototherapeutic agents can largely overcome the obstacle from the cell membrane for intracellular delivery. These strategies successfully transformed the weakness of phototherapy due to the enlarged inflammation into strength [94]. The release of the drug can be controlled under photodynamic therapy-induced inflammation, thereby largely endowing a good biosafety profile to untargeted normal tissues (Fig. 6d) [93]. Using multiple administrations of photosensitization or photothermal therapy can create a positive feedback loop in terms of inflammatory signals, further enhancing the recruitment of neutrophil membrane-coated nanoparticles in subsequent treatments. For instance, when using black phosphorus (BP) coated with neutrophil membrane for photothermal and photodynamic therapy, it provides a homogeneous and strong target signal, thereby achieving continuously improved therapeutic efficacy [95]. Similarly, modest radiation can also be an alternative to introduce signals that attract neutrophils for tumor-specific drug distribution. Radiotherapy can induce sterile inflammation, rapidly activating neutrophils for infiltration into the tumor site [43,49]. Despite efficient drug delivery, the curative effect might also be ascribed to the stronger photothermal effect and the heat activation of neutrophils for killing residual tumor cells via NETs [96]. The multistage delivery of photothermal agents into the deep tumor also benefits controlling the tumor recurrence [40]. Meanwhile, delivering photothermal agents by neutrophils can further prevent severe damage to the surrounding normal tissue by heat energy [97].

Overall, these innovative approaches demonstrate the versatility and potential of neutrophil-based nano-drug delivery systems in enhancing drug delivery to cancer cells and improving therapeutic efficacy.

4.1.3. Other inflammatory diseases

Targeting inflammation is a critical principle in the treatment of a variety of diseases from the standpoint of drug delivery, and neutrophil-based delivery strategies offer promising potential in this regard. Diseases including inflammatory bowel disease, rheumatoid arthritis, and bacterial infections are some of the diseases where neutrophil-based delivery strategies are commonly applicable [28]. Traditionally, inflammation targeting in drug delivery has focused on vehicles that bind to pro-inflammatory cytokines, which are upregulated during inflammatory responses [98]. While this approach has shown some success, the complexity of inflammatory processes can make effective and long-term targeting challenging. Treating inflammatory diseases with a single or a few anti-inflammatory molecules may not be sufficient to slow the disease progression effectively, underscoring the need for more robust targeting strategies. A multi-targeted approach can offer benefits, as it can address the complexity of the inflammatory cascade and target multiple pathways simultaneously. However, the complex system may also hinder its translation in clinical settings. Under such circumstances, Neutrophils, with their ability to interact with multiple molecules and cells involved in inflammation, present an attractive option for such multi-targeted strategies.

Neutrophils are inflammation-responsive cells that dominate the early stages of inflammation and mediate inflammation [99]. Neutrophils detect extracellular chemical gradients and move towards higher concentrations. Once the chemoattractant interacts with the receptor on the neutrophil membrane surface, the neutrophils undergo gradient sensing, polarization, and cell motility, enabling them to move intravascularly and extravascularly along a concentration gradient [75]. TNF-α receptor, IL-1β receptor, IL-6 receptor, lymphocyte function-associated antigen receptor (LFA-R), and CXCR2 on the surface of neutrophils are crucial for cytokine binding. These crucial receptors, which are mostly preserved after neutrophil membrane coating, endow nanoparticles with the ability to target the inflammation site and absorb pro-inflammatory cytokines and chemokines, including TNF-α, IL-1β, IL-6, and CXCL2, to alleviate inflammation and regulate the microenvironment [100]. Neutrophil membrane-coated nanoparticles can be used to target cytokine-activated fibroblasts, inflamed endothelial cells for anti-infection treatment [97]. The function of neutrophil membrane-coated nanoparticles crossing the endothelial barrier is further underpinned by adhering to inflamed endothelial cells and subsequent transmigration [101].

Arthritis and myocardial ischemic injury are two conditions closely related to inflammation, and neutrophil-based delivery strategies have shown promise in addressing their treatment challenges. In rheumatoid arthritis, activated neutrophils that have been attracted to the joint can stimulate synovial cells and induce their production of chemokines. The chemokines subsequently triggered the self-amplified recruitment of neutrophils. This positive feedback mechanism ensures the accumulation of neutrophil-like nanoparticles deep into the synovial fluid and cartilage [27]. Additionally, targeting peptides, such as R4F, can be anchored to the nanoparticles to specifically target the scavenger receptor, class B type 1 (SR-B1) on the macrophages in rheumatoid arthritis. This peptide mediates macrophage uptake for reprograming macrophage polarization, thus modulating the immune response, and reducing inflammation in the joint (Fig. 6c) [102]. The delivery of anti-inflammatory drugs to macrophages in the synovial fluid after systemic administration can also help reduce hepatotoxicity associated with undesired biodistribution of free drugs. In myocardial ischemic injury, endothelial progenitor cells are one of the commonly used strategies for cardiovascular repair. However, the limited accumulation of endothelial progenitor cells at the lesion site can hinder ideal therapeutic outcomes. To improve the infiltration of endothelial progenitor cells in the affected areas, neutrophils are utilized as carriers for nanoparticles. The nanoparticles are conjugated with CD34 antibodies that actively capture endothelial progenitor cells, thus enhancing the trapping of endogenous $CD34^{+}$ endothelial progenitor cells to the ischemic foci. Meanwhile, the superparamagnetic iron oxide encapsulated in nanoparticles allows for tracking the biodistribution of therapeutic agents using magnetic resonance imaging after intravenous administration [103].

While most strategies leveraging neutrophils as drug carriers take advantage of the maturation and activation of neutrophils to the inflammatory sites, the tropism of aged neutrophils back to bone marrow is also a potential property for drug delivery, especially for diseases such as osteoporosis and bone metastasis cancer. The life cycle of neutrophils involves several stages, maturation in the bone marrow, release into circulation, migration, and eventual death and removal by other cells. Mature neutrophils with high CXCR2 expression and low CXCR4 expression migrate from the bone marrow to the inflammatory site. After undergoing senescence and apoptosis, however, aged neutrophils reverse their expression of CXCR2 and CXCR4 and navigate back to bone marrow, where they can cross the bone marrow-blood barrier and deliver drugs to the bone marrow [104].

4.2. Precision engineering of neutrophil dynamics for drug delivery

Neutrophils can be recruited to sites of infection, injury, or tissue damage, which is a part of physiological responses toward inflammation. In addition to the natural inflammation during disease progression, signals related to inflammation can be introduced for neutrophil targeting. By using the inducers, which can be chemical compounds, biological agents, and physical forces, the activation and recruitment of drug-carrying neutrophils can be programmable.

To achieve better control and manipulation of neutrophil-based drug delivery systems, various moieties can be incorporated to influence the movement of therapeutics in vivo. Combining neutrophils with microbubbles and other motors can reinforce their adhesion ability and resistance to shear stress. Higher resistance to shear stress is particularly valuable for neutrophils to initiate adhesion in the circulating blood flow and transmigrate through endothelial cells. For example, a bionic microbubble-neutrophil composite has been designed for delivering therapeutics to atherosclerotic vulnerable plaques. Neutrophils, when combined with microbubbles and subjected to ultrasonication, showed firm attachment to the rupture-prone site of plaques with enhanced acoustic signals [105]. In another study, urease motors have been attached to neutrophils through biotin-based conjugation for propelled movement of the carriers. Urea present in the blood acts as a fuel for these motors, through enzyme catalysis, carbon dioxide, and ammonia have been produced, thereby promoting moving and decreasing transportation time [106]. For more precise manipulation of the movement, arrangement, and even rotation of neutrophils with single-cell precision, optical tweezers can be applied to the neutrophil-based delivery system. This technology enables the navigation to desired sites through a designated route in vivo [107]. The driving force enhances the ability of neutrophils to cross biological barriers like the blood vessel wall, and mechanical perturbations from optical tweezers activate the resting neutrophils simultaneously [108]. Additionally, by selectively delivering magnetic nanogels to neutrophils, neutrophil-based microrobots with self-propelling properties can be formed. These microrobots can accumulate around diseased sites under the control of a rotating magnetic field, allowing for active penetration of neutrophils across the blood-brain barrier for chemo-drug delivery [60]. All these techniques contribute to the precise control over the directional movement and activation of neutrophils, making them an effective carrier for targeted drug delivery.

4.3. Genetically engineering neutrophils for drug delivery

As the member to mediate the immune response, neutrophils can be further engineered and combined with nanotechnology to generate a multistage and complex combination treatment. Different from strategies either only take advantage of the homing and targeting of neutrophils as the drug carrier, or those genetically engineered neutrophils which might have limited therapeutic effect, some complex systems take full advantage of the inherited function of neutrophils and nanotechnologies using the methods in bioengineering. One common strategy to engineer immune cells for targeted and enhanced immune response is to use CARs. The first attempt to modify neutrophils with CARs was in 1998, in which the CAR-neutrophils were capable of target-specific cytolysis [109]. However, neutrophils are relatively less well characterized compared to T cells, NK cells, and macrophages. Thus, there were only a few reports on CAR-engineered neutrophils since then [110, 111]. From the perspective of the function, the neutrophil-mediated tumor-killing effect is mainly based on the phagocytosis after cytotoxic immune synapse formation, which might not be as specific and professional for robust immune defense as CAR-T cells. The limited development might also be due to the short lifespan of neutrophils and their resistance to genome editing. Also, the high possibility of neutrophils' apoptosis ex vivo prevents flexible engineering. Despite all the challenges, the successful engineering of neutrophils or differentiation from genetically engineered human pluripotent stem cells (hPSCs) inspired and enabled researchers to combine with neutrophil carriers [111]. Recently, Chang et al. designed a CAR-neutrophil-mediated

delivery system using hPSC-derived CAR-neutrophils to load mesoporous SiO_2 nanoparticles containing chemo-drug as a treatment for GBM. This innovative approach combined chemotherapy drugs with immunotherapy to address the heterogeneity of cancer-associated neutrophils within the TME impacting their anti-tumor and pro-tumor phenotypes which contribute to drug resistance (Fig. 7) [45]. The treating dual-drug loaded, CAR-engineered neutrophils lead to a significant reversal of the immunosuppressive TME and inhibition of tumor growth.

4.4. Other applications

Diagnosis is one emerging application for combining neutrophil-based nanomedicine with other modalities beyond drug delivery. By loading probes for diagnosis, it is possible to detect diseases, especially related to the recruitment of neutrophils. Meanwhile, it can also decrease the background noise of the signals, help to reduce the rapid clearance, and overcome challenges, including photobleaching and diffuse scattering, thus improving the precision and accuracy for tracing and diagnosis. Many diagnostic agents can be combined with the neutrophil-based cell delivery system. For example, magnetic resonance imaging (MRI), an extensively well-studied technique, can be applied not only for studying the distribution and behavior of neutrophils in the designed delivery system, but also serve as the biomimetic theragnostic platform for glioma after surgery. Neutrophils as terminal-differentiated cells have superior advantages to carry MRI contrast agents as the signal would not decrease and be diluted due to the proliferation [72]. Expanding this idea, Sun et al. have developed in-situ engineering for the delivery of inflammation-induced chemiluminescence to recognize inflamed tissues for diagnosis (Fig. 8a) [47]. This chemiluminescent probe can first be carried by neutrophils to the inflammatory site, followed by the release of caged photons only upon inflammation for visualization, further decreasing the background signal. Similar in employing neutrophils' tropism to inflammation, Yu et al. combined neutrophil-coated nanoparticles with a Ru complex in the form of $[Ru(bpy)_2(tip)]^{2+}$ (RBT) for early diagnosis and treatment of osteoarthritis (Fig. 8b) [31].

5. Challenges and future perspectives

Neutrophil-based nano-drug delivery systems have shown great promise for expanding the range of nanoparticles used for drug delivery, overcoming the limitations imposed by the properties of nanoparticles, such as material, size, and surface charge. Neutrophil-based nano-drug delivery systems offer alternative approaches for diverse disease treatments, albeit predominantly at the preclinical stage. They can significantly extend the retention time of therapeutics, endow biomimetic property, reduce nanoparticle clearance in the bloodstream, and enhance their targeting ability. In turn, nanoparticles can further reinforce the native chemotactic functions of neutrophils, boosting their recruiting process for stronger delivery capabilities.

However, to fully harness their potential, certain restrictions and challenges must be addressed for the successful implementation of a neutrophil-based drug delivery system. The safety of the drug delivery system is of utmost importance, which is also one of the key challenges for translating those systems to clinical applications.

Safety considerations encompass potential issues stemming from the stimulation or infusion of neutrophils. The utilization of allogenic neutrophils, although potentially more accessible for scalability, raises concerns about rejection issues and immune response [112]. Additionally, the intricate interplay of neutrophil subtypes and their dual roles in disease adds additional complexity, as administered living neutrophils may be susceptible to reprogramming if not appropriately restricted [45]. Considering the dual role of neutrophils in inflammation, maintaining a delicate balance between their damaging and reparative functions is crucial [113]. However, evidence suggests that chronic inflammation or tissue damage may arise from aberrant neutrophil recruitment and activation [114]. While many nanotherapeutic delivery systems employing whole neutrophils are designed for diseases with inflammation and substantial neutrophil infiltration, the delivery process would also involve the dying of neutrophils at targeted sites, releasing enzymes and creating an environment that attracts more neutrophils and immune cells. This could potentially perpetuate an overcrowded inflammatory microenvironment, fostering chronic inflammation [115]. Interactions between neutrophils, platelets, adaptative immune cells, and macrophages further contribute to sustained inflammation [116]. The nuanced exploration on the potential for chronic inflammation post-treatment will enhance our understanding of the long-term effects of neutrophil-based nano-drug delivery systems.

Concerns on the off-target effects persist in neutrophil-based drug deliver also exist. Currently, the release mechanism for utilizing whole neutrophil as nanotherapeutic carrier is mainly relying on the NETosis and degranulation, the natural function of activated neutrophils. Unwanted drug release might occur in the sites where clearance of aged neutrophils take place, including liver, spleen, and bone marrow, or the places with acute or chronic inflammation. To further mitigate off-target

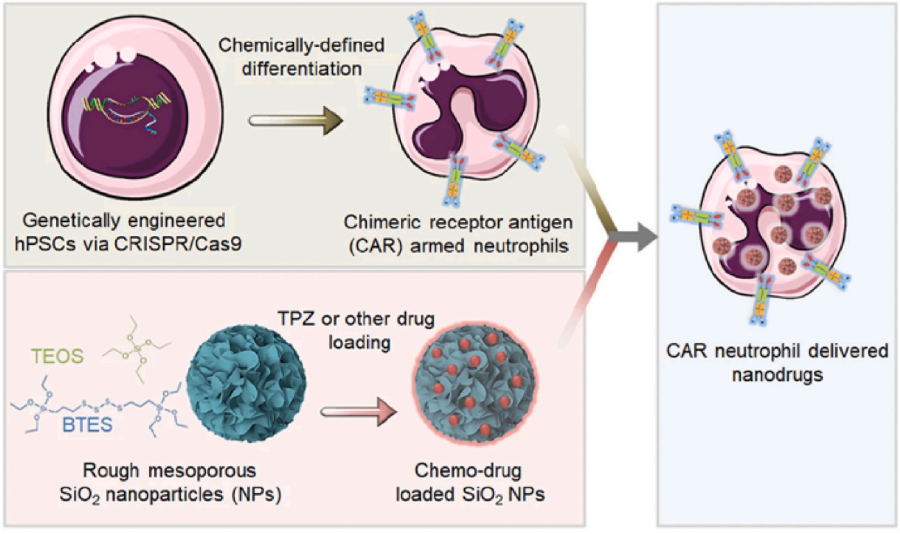

Fig. 7. Engineered CAR-neutrophils for nanodrug delivery. Reproduced with permission [45]. Copyright 2023, Springer Nature.

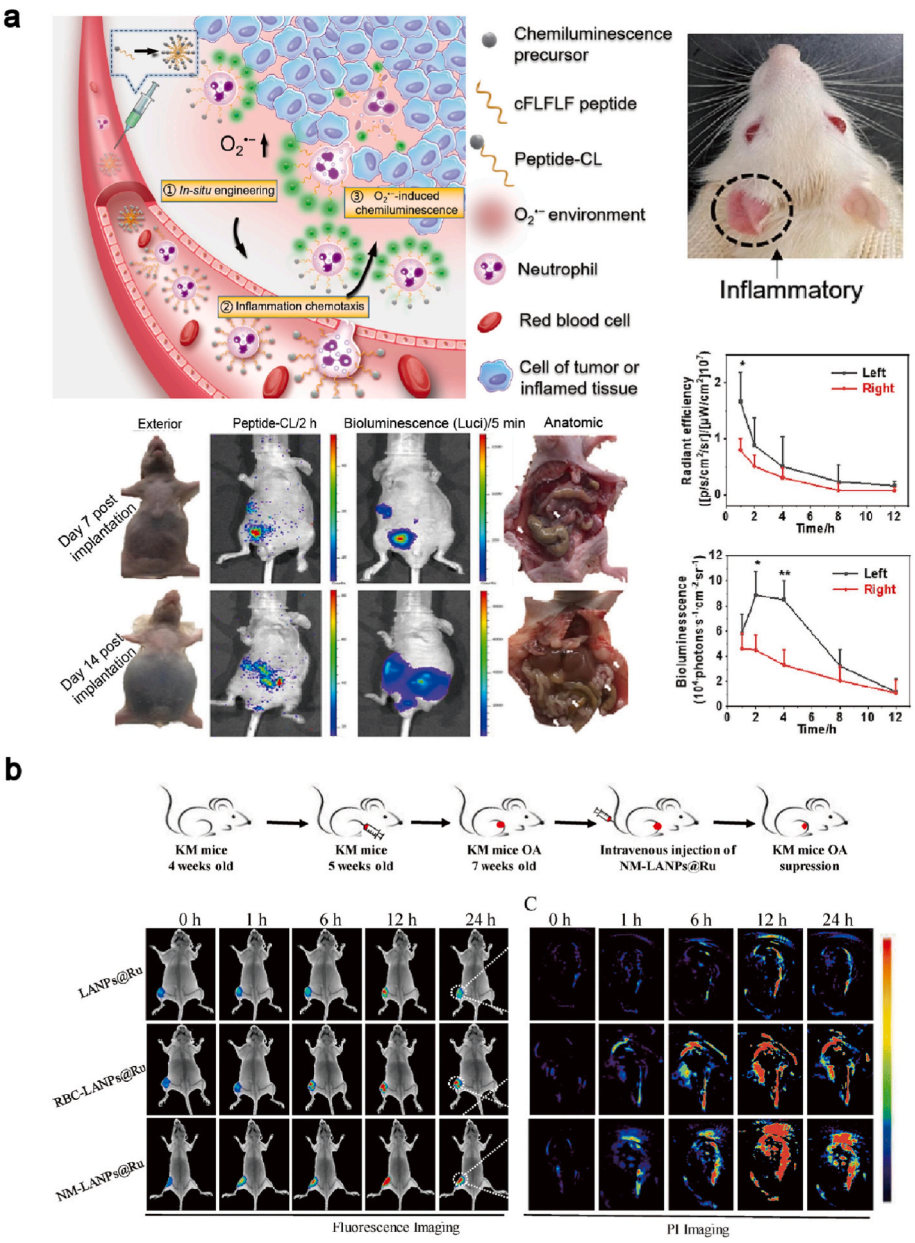

Fig. 8. Engineering neutrophils and other applications. **a)** The imaging of O_2 radical dots for colorectal cancer peritoneal metastasis and ear swelling with *in-situ* assistance of neutrophils. Reproduced with permission [47]. Copyright 2023, Elsevier B.V. **b)** Ru complex coated by neutrophil membrane for early diagnosis of osteoarthritis. Reproduced with permission [31]. Copyright 2022, Royal Society of Chemistry.

effects, technologies employing nanomaterials responsive to specific signals with controlled release profile are integrated into the neutrophil-based drug delivery system. Meanwhile, Rigorous evaluation of side effects stemming from off-target drug release is essential.

As for *in-situ* hijacking of neutrophils, one of the key challenges and limitations associated with this approach pertains to the efficiency of nanoparticles in targeting circulating and activated neutrophils, as well as the extent of internalization by these neutrophils. Addressing this challenge necessitates a rational design of nanoparticles to enhance both targeting and internalization processes, thereby optimizing delivery efficiency. An additional consideration revolves around the timing of nanoparticle administration. Neutrophils exhibit distinct roles during the progression of diseases, influencing their potential for drug delivery. The dynamic nature of inflammation across different disease stages further complicates the recruitment of neutrophil-based drug delivery systems. It is also necessary to evaluate the heterogeneity of neutrophils in different patients while research have showed reduced neutrophil migration from the aged host [117]. The differences in diseases, stages, and patients potentially lead to varied therapeutic outcomes, underscoring the need for a tailored approach based on specific disease contexts. Currently, most pre-clinical studies have used mice model to evaluate the safety profile and treatment efficacy of neutrophil-based drug delivery systems. However, mouse neutrophils only count for 10–15 % of circulating leukocytes, while 50–70 % are neutrophils in human blood [118]. The differences between human and mouse immunology also pose limited validation when using mice models to accurately reflect human neutrophil behaviors.

Addressing scalability and reproducibility challenges is contingent upon overcoming the limited source of neutrophil or their membrane, as well as their short lifespan. Continuous and successive administration of therapeutically loaded neutrophils may circumvent this obstacle. Some studies have also explored the use of human pluripotent stem cells induced into neutrophils using stage-specific chemical stimulators [110]. Others have exploited myeloid cells to engineer artificial

neutrophils with outstanding functional performance [119]. These innovative approaches provide a promising avenue for achieving improved scalability and reproducibility, offering the potential for off-the-shelf neutrophil-based systems.

The success of neutrophil-based drug delivery relies on fully exploiting their native functions. Difficulty in neutrophil engineering, low loading efficiency, and unspontaneous chemotactic motion need optimization to maximize their therapeutic benefits. In regard to the specificity, achieving tissue-targeting is feasible due to the presence of inflammation signals in the microenvironment of disease sites. Yet, enhancing the specificity of the delivery system to single cell types or specific phenotypes remains a promising avenue for further exploration. More pre-clinical are necessary to validate the safety and efficacy of different diseases, and risk-mitigation strategies should be developed to overcome these challenges. Artificial intelligence (AI) technology can be used for harnessing the modality of the complex neutrophil-based platform. For instance, CAR-neutrophils phenotypes with various signaling motifs can be decoded to optimize CAR design for increased cytotoxicity [120]. By using machine learning, it is now possible to quantitate the process of neutrophil NETosis. providing more information on the payload release [121]. It is important to choose drugs and materials that would not impact the physiological functions of neutrophils that are necessary for successful delivery after those nanotherapeutics are internalized. A high-throughput screening platform is needed to find and optimize current strategies. Moving forward, a focus on understanding the unique functions of neutrophils in disease contexts is essential to enhance the design and efficacy of these systems. By gaining a comprehensive understanding of the mechanisms underlying neutrophil migration and tropism, new opportunities can be identified for developing more targeted drug delivery systems. Advances in neutrophil engineering like genetic manipulation and other biotechnologies can pave the way for personalized and precise medicine. Researchers are also actively working to achieve higher loading efficiency, develop simpler operation methods, and reduce the overall cost and time required for scaled-up production.

6. Conclusions

In conclusion, this paper summarizes current progress in combining neutrophils and nanomedicine for drug delivery, covering underlying mechanisms to versatile applications. Promising results have been shown in utilizing neutrophils for precise, robust, and controllable drug delivery by either coating nanoparticles with their membranes, loading nanoparticles inside living cells, or engineering CAR-neutrophils. Neutrophil-based treatments have become a promising direction for bio-inspired drug delivery systems.

Ethics approval and consent to participate

This review article does not require any ethical approval or allied consents for publication.

CRediT authorship contribution statement

Sichen Yuan: Writing – review & editing, Writing – original draft, Conceptualization. **Quanyin Hu:** Writing – review & editing, Conceptualization.

Declaration of competing interest

We declare no conflict of interest.

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