Therapeutics

FORUM REVIEW ARTICLE

Exploiting Oxidative Microenvironments in the Body as Triggers for Drug Delivery Systems

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Abstract

Significance: Reactive oxygen species and reactive nitrogen species (ROS/RNS) play an important role in cell signaling pathways. However, the increased production of these species may disrupt cellular homeostasis, giving rise to pathological conditions. Biomaterials that are responsive to ROS/RNS can be strategically used to specifically release therapeutics and diagnostic agents to regions undergoing oxidative stress. *Recent Advances:* Many nanocarriers intended to exploit redox micro-environments as triggers for drug release, summarized and compared in this review, have recently been developed. We describe these carriers' chemical structures, strategies for payload protection and oxidation-selective release, and ROS/RNS sensitivity as tested in initial studies. *Critical Issues:* ROS/RNS are unstable, so reliable measures of their concentrations in various conditions are scarce. Combined with the dearth of materials shown to respond to physiologically relevant levels of ROS/RNS, evaluations of their true sensitivity are difficult. *Future Directions:* Oxidation-responsive nanocarriers developed thus far show tremendous potential for applicability *in vivo*; however, the sensitivity of these chemistries needs to be fine tuned to enable responses to physiological levels of ROS and RNS. *Antioxid. Redox Signal.* 21, 730–754.

Introduction

REACTIVE OXYGEN SPECIES (ROS) and reactive nitrogen species (RNS) found in living organisms are produced as by-products of biochemical reactions occurring within the cell. Some of the most common ROS and RNS include H_2O_2 (hydrogen peroxide), $O_2^{\bullet-}$ (superoxide), $^{\bullet}OH$ (hydroxyl radical), OCl^- (hypochlorite ion), NO (nitric oxide), and $ONOO^-$ (peroxynitrite). Due to their inherently reactive nature, ROS and RNS degrade rapidly by interacting with surrounding molecules, covalently modifying them. Antioxidants such as glutathione (GSH), citrulline, vitamin E, and polyphenols, along with enzymes such as superoxide dismutase (SOD), catalase, and GSH peroxidase (GPX), scavenge and quench ROS (158). At low physiological levels of ROS and RNS, chemically modified biomolecules (proteins and lipids) serve as key modulators of intra- and extracellular signaling pathways [extensively reviewed in (42, 57, 180, 182)]. At higher levels, ROS and RNS give rise to oxidative stress, a condition in which ROS/RNS concentrations exceed cells' antioxidant capacity. While oxidative stress is beneficial in processes such as inflammation (*e.g.*, immune cell-mediated destruction of microbial agents), it can also be detrimental to cells, as it induces irreversible changes to biomolecules. These changes, such as carbonylation, glycation, S-glutathiolation, and S-nitrosylation of proteins, have been implicated in the pathogenesis of diseases, including Alzheimer's and diabetic complications (24, 128, 151). Altered ROS regulation as a result of aging (108) may contribute to increased risk for oxidative stress-associated disorders. Lipids of cellular membranes are also damaged by oxidative conditions, giving rise to unstable lipid hydroperoxides (9). Chronic oxidative stress promotes breaks in double-stranded DNA [especially mitochondrial DNA (mtDNA)], leading to mutations linked with mitochondrial disorders (97). Chronic oxidative stress is, thus, considered an important contributing factor to the etiology of diseases such as cancer, neurodegeneration, and cardiovascular dysfunction.

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The unique redox microenvironment of cells and tissues experiencing oxidative stress can be exploited for the delivery of therapeutics and diagnostics agents *via* nanoscale carriers that are tailored to release their contents in conditions of oxidative stress. Current chemical mechanisms involved in biomaterials with potential of ROS-induced degradation or release have been reviewed in detail in a recent review (112). Drug delivery *via* nanoparticles promises to revolutionize human health by broadening the range of usable drugs and, with disease-specific engineering, reducing dosages and limiting side effects. Nanoparticles with hydrophobic cores enable the delivery of drugs that are poorly water soluble, which are otherwise difficult to administer intravenously at effective concentrations; other nanoscale formulations enable the delivery of proteins or peptides that are sensitive to proteolytic degradation, if left unprotected. The second advantage lies in the large surface area of nanoparticles, which enables the attachment of many antibodies, aptamers, or peptides that promote uptake by tissues or tumors that overexpress their cognate receptors, concentrating imaging signals or minimizing drug side effects. Using a slightly different approach, ''bioresponsive'' nanovehicles can be customized to respond to a stimulus (either characteristic of disease, for example, acidic pH, or user controlled, for example, near infrared light) by releasing cargo. Oxidation-sensitive nanovehicles, therefore, represent such a class of ''smart'' drug carriers that should target the delivery of therapeutics and diagnostic agents to regions experiencing high oxidative stress. To achieve this goal, potential ROS-responsive carriers may incorporate one of several strategies to translate a reaction with ROS to macroscopic physical changes in the nanocarrier that enable the release of contents. Such strategies include degradation, leading to swelling and dissolution of the carrier or a switch from hydrophobic to hydrophilic character of the nanocarrier material, causing complex disassociation.

In this review, we first briefly describe cellular sources of ROS/RNS and pathological conditions arising from oxidative stress. In order to explain the challenges inherent in assessing the sensitivity of ROS-responsive nanocarriers, we then discuss strategies for measuring ROS/RNS. Finally, we present a summary of the various chemical systems (micelles, polymeric nano/microparticles, and supramolecular hydrogels) developed to date that can respond to altered oxidative microenvironments.

Cellular Sources of ROS/RNS

Diseases involving oxidative stress result from alterations in cellular mechanisms regulating redox homeostasis. In order to design strategies to combat oxidative stress or to harness the unique redox microenvironments for the purpose of drug delivery, it is important to understand the cellular sources of ROS/RNS in order to ensure that they reach the appropriate subcellular location. Some of the main organelles involved in ROS/RNS production are described next, along with the enzymes involved in ROS/RNS production and scavenging (Table 1).

Mitochondria

Mitochondria are the primary sites for oxygen metabolism and account for the consumption of 85%–90% of cellular oxygen (161). Mitochondrial respiration produces primarily superoxide and hydrogen peroxide. Superoxide radicals are produced by Complex I and III of the electron transport chain (ETC) in the intermembrane space (15, 181) and ETC flavoproteins in the matrix (168). Superoxide is scavenged by the manganese SOD2 in the mitochondrial matrix to produce H_2O_2, which can freely diffuse across membrane bilayers (193). Another source of cytosolic H_2O_2 is monoamine oxidase, a non-ETC enzyme that metabolizes neurotransmitters and dietary monoamines, which are present in the mitochondrial outer membrane (163). This H_2O_2 is consumed in the cytosol by catalase (67) and in the mitochondria, by GPX and thioredoxin peroxidase (27, 56). Alternatively, H_2O_2 can react with iron clusters that are abundant in the mitochondria *via* Fenton chemistry to form hydroxyl radicals (71).

Mitochondria also contribute to the production of RNS, also potent oxidizers, by the action of mitochondrial nitric oxide synthase (mtNOS), which produces nitric oxide (NO) (59). Nitric oxide can react with mitochondrial superoxide and produce peroxynitritite ($ONOO^-$) (59). Peroxynitrite can oxidize proteins, especially mitochondrial enzymes such as the components of the ETC and SOD2, or cause calcium release, changing mitochondrial permeability (59).

Oxidative stress from mitochondrial dysfunction has been linked to several neurological disorders such as Alzheimer's and Parkinson's (see next section for their pathophysiology). Some of these disorders result from dysfunction of non-mitochondrial proteins; for example, in Friedreich's ataxia, mutations in an iron-binding protein cause mitochondrial iron overload, which damages mitochondrial proteins that usually reduce ROS (197). Other rare diseases result from mutations in the mitochondrial DNA (mtDNA) that affect the function of ETC, which cause respiratory defects and enhanced ROS production (174).

Peroxisomes

Peroxisomes are specialized organelles that function primarily in the catabolism of long-chained and branched-chain fatty acids by β-oxidation. Peroxisomal enzymes also detoxify and metabolize compounds that are poorly soluble in water and lipids (5). The primary ROS byproduct of peroxisomal enzymes (*e.g.*, acyl CoA oxidase, urate oxidase, and amino acid oxidase) is H_2O_2; however, superoxide and nitric oxide are also produced by xanthine oxidase and NO synthase, respectively (5). To protect against oxidants, peroxisomes also contain enzymes that scavenge ROS. These include catalase, SOD, peroxiredoxin, epoxide hydrolase, GSH S-transferase, and several peroxisome-specific integral membrane proteins (peroxisomal membrane protein 2, Mpv-17, and Mpv-like protein) (5). If peroxisome proliferation is stimulated (*e.g.*, by pharmacological agents) or peroxisomal ROS-scavenging enzymes are mutated, this results in excessive ROS production and oxidative stress, which may lead to disease. Imbalances in the homeostasis of ROS in peroxisomes have been linked to diseases such as cancer, neurodegeneration, and type 2 diabetes (61).

Plasma membrane/extracellular space and phagosome

ROS are also produced to combat infectious disease: Phagocytes, especially neutrophils and monocytes, rapidly

TABLE 1. ENZYMES INVOLVED IN THE PRODUCTION OR CONSUMPTION OF REACTIVE
OXYGEN SPECIES AND REACTIVE NITROGEN SPECIES

Enzyme	Reaction	Localization and tissue abundance	Ref.
Copper-zinc superoxide dismutase (Cu/ZnSOD or SOD1)	$2O_2^{\bullet-} + 2H^+ \rightarrow O_2 + H_2O_2$	Cytoplasm, nucleus, lysosomes, and mitochondria	(31, 63, 129, 147)
Manganese SOD (MnSOD or SOD2)		Mostly mitochondria	(92, 164, 193)
Extracellular SOD (ECSOD or SOD3)		Extracellular space; 10 times higher activity in vascular walls	(65, 170)
Catalase	$2H_2O_2 \rightarrow 2H_2O + O_2$	Peroxisomes; mostly in liver parenchyma and renal proximal tubule	(29, 30, 86)
Myeloperoxidase (MPO)	$H_2O_2 + Cl^- \rightarrow HOCl + OH^-$	Lysosomes of polymorphonuclear neutrophils	(96, 99)
Glutathione peroxidase	$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O$	GPX1, cytoplasm of most tissues; GPX3, plasma	(7, 35, 36, 133, 173)
Xanthine oxidase	$Xanthine + H_2O + O_2 \rightarrow$ uric acid $+ H_2O_2$	Cytoplasm in liver, small intestine, and mammary gland	(52, 109, 119, 135, 189)
NADPH oxidase	$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{-\bullet} + H^+$	Plasma and phagosome membranes in neutrophils	(16, 73, 156)
Mitochondrial cytochrome C I,II and II	electron transport chain	Inner membrane of mitochondria	(14, 22, 60)
Monoamine oxidase	$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$	Outer membrane of mitochondria, plasma	(14, 22, 60, 83, 172, 199)
D-Amino acid oxidase	D-amino acids $+ O_2 + H_2O \rightarrow$ α-keto acid $+ NH_3 + H_2O_2$	Peroxisomal membranes in liver, kidney, and brain	(41, 69, 131)
Nitric oxide synthase 2, inducible (iNOS2)	L-arginine $+ 3/2$ NADPH $+$ $3/2H^+ + 2$ $O_2 \rightarrow$ L-citrulline $+$ NO $+ 3/2$ NADP$^+ + 2H_2O$	Peroxisome, plasma membrane, and cytoplasm	(89, 100, 107, 169)
[a]Fenton-type mechanism, iron redox-cycling	Fe^{2+} (ligand) $+ H_2O_2 \rightarrow Fe^{3+}$ (ligand) $+ OH^- + HO^{\bullet}$	Endoplasmic reticulum (under normal conditions)	(79, 118, 179)

[a]Nonenzymatic.
NADPH, nicotinamide adenine dinucleotide phosphate.

secrete superoxide and hydrogen peroxide to kill invading pathogens (140). The recognition of foreign particles triggers the assembly of the NADPH oxidase (NOX) complex in the phagocytic plasma membrane, which produces extracellular superoxide that is rapidly converted to hydrogen peroxide (21). After phagocytosis, the NOX complex remains on the membrane of intracellular phagocytic vesicles, producing an ROS-rich environment inside the vesicles. The fusion of phagosomes with myeloperoxidase-rich azurophilic granules in neutrophils results in the formation of hydroxyl radical and hypochlorous acid, which is pivotal in killing invading pathogens (21).

NOX1 is also expressed on the plasma membrane of non-phagocytic cells (e.g., colonic epithelium, vascular smooth muscle) and is responsible for creating an oxidative environment in the extracellular space (28). Alternately, superoxide may also react with nitric oxide (produced by NOS) and form the highly oxidizing peroxynitrite, which can, in turn, induce lipid peroxidation, causing damage to the cell membrane (28). The accumulation of extracellular hydrogen peroxide can also be attributed to its diffusion from an intracellular source (mitochondria) through the lipid bilayer or membrane-associated channels such as aquaporins (28). Cell signaling pathways at the cell surface (e.g., receptor-ligand interactions) also generate hydrogen peroxide. The production of hydrogen peroxide in response to various receptor-ligand interactions was measured in cells and was found to be enhanced when a cognate receptor-ligand pair was chosen (48). Modulators of extracellular ROS/RNS include extracellular SOD (EC-SOD), which converts superoxide anion to hydrogen peroxide, GPX3, and thioredoxin reductase (28). However, the abundance of these anti-oxidants is much lower in the extracellular space compared with the intracellular environment. Alterations in the expression of levels of NOX1 were found to be elevated in certain types of cancers, while the levels of antioxidants such as GPX3 were down-regulated in others (28).

Diseases Involving High ROS/RNS Concentrations

ROS-responsive chemistries could be used to develop diagnostics or drug carriers for any disease involving significantly increased concentrations of ROS. Here, we describe the major categories of such diseases, including the pathophysiology of several examples and how they relate to the development of ROS-responsive technologies.

Inflammatory diseases

Systemic inflammatory response syndrome. Systemic inflammatory response syndrome (SIRS), a hyperactive immune response, most commonly results from severe infection (such cases are referred to as sepsis), burns, traumatic injury, ischemia, and hemorrhage. SIRS symptoms include hyper- or hypothermia, tachycardia, hyperventilation, and leukocytosis or leukopenia (44). This aggravated systemic immune response is characterized by the release of pro-inflammatory mediators such as tumor necrosis factor alpha (TNFα), interleukin (IL)-1, IL-6, and colony-stimulating factors, which activate and recruit polymorphonuclear leukocytes (PMNs), monocytes, and macrophages (46). These cytokines also increase vascular permeability, making organs susceptible to the destructive effects of phagocytes. Recruited PMNs, especially neutrophils, generate large quantities of superoxide and hydroxyl radicals, along with hypochlorous acid in the processes of phagocytosis and degranulation as described in an earlier section (44). In addition, activated neutrophils move within tissue space by secreting extracellular matrix (ECM)-degrading enzymes (metalloproteases, collagenases, and elastases), which are the key contributors to organ failure (46). Given the high degree of neutrophil recruitment and their ability to produce huge amounts of ROS/RNS, SIRS models likely provide an excellent system to evaluate novel ROS-responsive materials.

Chronic obstructive pulmonary disease. Chronic obstructive pulmonary disease (COPD) consists of chronic bronchitis or emphysema and can progress into respiratory failure. COPD is caused by repeated exposure to oxidants in cigarette smoke and pollutants, rich in alkyl, alkoyl, and peroxyl organic-free radicals in addition to superoxide and nitric oxide, which create an oxidizing environment in the lung alveoli (53). These irritants also trigger an inflammatory response culminating in the infiltration of neutrophils and macrophages in lung tissue; viral airway infections exacerbate the disease. Patients with COPD have higher levels of neutrophils, macrophages, and CD8$^+$ cells in their peripheral airways than nonsmoking controls (126, 155). Chronic inflammation results in damage to the alveolae due to destruction of ECM by neutrophil elastase and ROS generated by activated, degranulating neutrophils (91, 137). Imbalances in lung remodeling and tissue regeneration due to increased apoptosis and autophagy of airway epithelium prevent the restoration of normal lung function (84).

If ROS concentrations are significantly enhanced, ROS-responsive materials could prove useful in the diagnosis of COPD, as the alveoli become oxidatively stressed early in disease progression and COPD is now diagnosed based on lung function, after damage has occurred.

Rheumatoid arthritis. Rheumatoid arthritis (RA) is characterized by inflammation of the synovial membrane followed by cartilage and bone damage. RA consists of an autoimmune response, usually against citrullinated vimentin and fibrinogen; environmental triggers, such as smoking, periodontitis, and exposure to microbes such as Epstein-Barr virus, cytomegalovirus, and *Escherichia coli*, are associated with enhanced citrullination of proteins (6, 106, 124, 165, 192). Autoantigen-sensitized T cells (T$_H$17 cells) and B cells in the synovium produce chemokines (IL-17 and TNFα) that recruit dendritic cells, macrophages, and granulocytes (26, 145). These leukocytes release ROS/RNS, along with cytokines, prostaglandins, and metalloproteases, inflaming the joint (25, 32). Metalloprotease activity enables synoviocyte migration out of the joint, which contributes to damage to the cartilage (114). The chemokine-rich synovial environment also promotes osteoclastic differentiation of macrophages; osteoclasts demineralize cartilage and bone (102, 176). Targeting RA therapeutics to the synovium would reduce side effects, such as the increased susceptibility to infection with TNFα inhibitors.

Atherosclerosis. Atherosclerosis is characterized by arterial lesions or plaques that are composed of cholesterol, smooth muscle cells, ECM, and leukocytes. Unstable (or vulnerable) atherosclerotic plaque is a major risk factor for sudden coronary death and it originates from the deposition of apo-B-containing lipoproteins on arterial walls (58, 195). This fatty deposition is infiltrated and phagocytosed by macrophages and dendritic cells, often giving rise to "foam cells" (103). On accumulation of excessive free cholesterol, many foam cells undergo apoptosis (159). The inability of macrophages to clear the apoptotic population from the plaque creates necrotic lesions that are susceptible to rupture (157). Furthermore, macrophages produce ROS and cytokines that are involved in the recruitment of additional leukocytes and the proliferation of smooth muscle cells into the lesion (175). ROS production has been shown to activate matrix metalloproteinases (MMPs), which promotes the destruction of the collagen matrix surrounding the plaque, further destabilizing it (148). The secretion of thrombogenic factors by macrophages on plaque rupture recruits additional platelets to form a thrombus, increasing the risk of embolism (64, 175).

Early, reliable diagnostics for vulnerable plaque would make an enormous health impact, identifying patients in need of aggressive early therapy and potentially preventing heart attacks. ROS-responsive materials may provide such a diagnostic if they are sufficiently sensitive.

Neurodegenerative diseases

The overproduction of ROS has been observed in many neurodegenerative disorders, including Parkinson's, Alzheimer's, Huntington's disease, and amyotrophic lateral sclerosis (ALS). Though these diseases cause distinct patterns of neurodegeneration (first affecting dopaminergic motor areas in Parkinson's and Huntington's, the frontal lobe in Alzheimer's, and motor neurons in ALS), similar factors, discussed next, have been shown to contribute to mitochondrial dysfunction in these diseases.

Environmental factors. The use of pesticides in developing countries has been suggested as a key risk factor for the incidence of Parkinson's disease (PD) (18). Pesticides such as

dipyridyl herbicides (paraquat), organochlorides (dieldrin), and dithiocarbamates (maneb) have been shown to interfere with the mitochondrial ETC, producing higher levels of ROS (12, 98, 203).

Mutations in genes involved in ROS regulation. Both ALS and Parkinson's have been linked to mutations in genes that regulate ROS levels, in either the cytosol or mitochondria. Many cases of familial ALS are associated with mutations in SOD1 (153), and elevated ROS and ROS-induced damage of motor neurons has been reported in postmortem ALS tissue and biopsy samples from ALS patients (8, 10). However, ALS etiology is complex and involves a combination of events, including elevated ROS, protein misfolding, endoplasmic reticulum (ER) stress, autophagosomal dysfunction, and impaired cytoskeletal rearrangement. Similarly, mutations in mitochondrial kinase PINK1, and loss of Parkin and oxidative stress sensor DJ-1 activities have been implicated in the pathogenesis of PD, with an increase in oxidative stress being one of the contributing factors for disease progression (78, 119, 146).

Inflammation. Activation of astrocytes and microglia are common to all neurodegenerative disorders. In Alzheimer's and Huntington's, insoluble protein aggregates and dying neuronal cells serve as signals for the activation and recruitment of immune cells (microglia) (72), which promote neurotoxicity by releasing additional ROS and RNS (38, 136). High ROS levels have also been observed in other protein aggregation diseases such as Alzheimer's and Huntington's disease in which the accumulation of misfolded protein aggregates has been postulated to interfere with mitochondrial function and the production of free radicals from aggregate interactions with transition metal ions such as iron, copper, and zinc (66).

Cancer

Cancer is a complex disease that is characterized by unregulated cell division of abnormal cells, which have the potential to migrate and spread to distal parts of the body. Many types of cancers are distinguished by high levels of ROS/RNS (171), which is attributed, in part, to erratic signaling and abnormal levels of cytokines and growth factors (90, 117). For example, TNFα and IFNγ signaling in certain tumors have shown to enhance levels of hydrogen peroxide and nitric oxide (117). Similarly, increased expression of oncoproteins Bcr-Abl (tyrosine kinase) (94), c-Myc, and decreased p53 activity has been correlated with increases in tumor ROS levels (184). Enhanced NOX1 expression in cancer cells has also been implicated as a major contributor to extracellular ROS; differences in NOX1 expression correlate with the degree of transformed phenotype (28).

Evidence that the overproduction of ROS/RNS contributes to the progression of cancer comes from several studies of genetically modified mice in which the expression of ROS-regulating enzymes is altered. Mouse knockouts of ROS-quenching enzymes (SOD1, SOD2, and GPX) have greater susceptibility to carcinogenesis (37, 54, 187). Transgenic mice that are deficient in SOD1 show signs of oxidative damage (lipid peroxidation and DNA damage) in the liver and develop hepatocellular carcinoma (54). The significance of ROS/RNS in cancer is further highlighted by studies demonstrating that the overexpression of EC-SOD inhibits melanoma *in vivo* and breast cancer growth *in vitro* (177, 194). Additional experiments using cancer cell lines have shown a link between ROS/RNS levels and invasiveness, presumably due to ROS/RNS-induced activation of MMPs that enable migration (28).

Detection of ROS/RNS in Biological Systems: Strategies and Challenges

Ideally, the summary cited earlier would include a comparison of ROS levels in various disease conditions; however, a reliable measurement of concentrations of these unstable species remains extremely challenging. The major challenge in measuring levels of ROS and RNS in real time is that these molecules are highly labile and therefore have a very short half life; whereas various ROS sensors have distinct sensitivities, making comparisons across studies difficult. Further, most indicators do not provide quantitative readouts. Most of the studies cited measure H_2O_2, as it is one of the most abundant and stable types of ROS. Some of the techniques to measure H_2O_2 have been reviewed here (75). Since what constitutes "biologically relevant" levels of H_2O_2 has been reviewed elsewhere (20, 80), we will focus here on tools for ROS/RNS detection; a more comprehensive list is provided in Table 2.

Chemical probes

Dichlorodihydrofluorescein diacetate. Dichlorodihydrofluorescein diacetate (H_2DCFDA) is cleaved by cellular esterases and oxidized by ROS to produce fluorescence (40, 154). H_2DCFDA and its derivatives, which freely cross cell membranes, have been widely used as ROS sensors in cell culture models (74, 116). As shown by a recent study in *Caenorhabditis elegans*, H_2DCFDA enables the study of real time *in vivo* ROS generation across tissue types; in this case, in response to challenge with *Salmonella enterica* (160). Although very popular, one of the main disadvantages of these dyes (as with other chemical probes) is that they cannot be targeted to a specific sub-cellular compartment for monitoring local changes in ROS/RNS. Moreover, they respond to a broad range of ROS/RNS and are susceptible to artifacts due to nonspecific signal amplification from photo-oxidation and reactions with heme-containing compounds (13, 141).

Amplex red. Amplex red is often used to assay mitochondrial respiration and the oxidative burst of neutrophils in suspension cultures (33, 134). Amplex red is readily oxidized to resorufin, which is highly fluorescent (134). While amplex red, unlike H_2DCFDA, is specific for H_2O_2 and can detect it at very low levels (50), it is cell impermeable and, thus, cannot be used to measure H_2O_2 levels intracellularly (50). In addition, it is susceptible to oxidation by reduced GSH and pyridine dinucleotides in the presence of NADH, creating artifacts (191).

Genetically encoded proteins

Redox-sensitive yellow and green fluorescent proteins (rxYFP and roGFP). rxYFP and roGFP were designed to noninvasively measure changes in the redox potential of cells

TABLE 2. COMMON EXAMPLES OF PROBES USED TO DETECT ROS IN CELLS OR *IN VIVO*,
INCLUDING SMALL MOLECULES, PROTEINS, AND NANOSTRUCTURES

ROS sensor	Mechanism	Sense	Readout[a]	Ref.
roGFPs	Oxidation of adjacent cysteine residues changes fluor. maximum	GRXs	Ex/Em: 400/490–510 nm	(82, 87, 130)
HyPer	Oxidization of adjacent cysteine residues changes fluor. maximum	H_2O_2	Ex/Em: 420/500–516 nm	(11, 70, 127)
Redoxfluor	Oxidation of cysteines in fragment of yeast redox sensor causes loss of FRET between cerulean and citrine	Redox potential of glutathione	Em ratio 527/476nm	(201)
DCFH-DA	Oxidation after esterase-mediated cleavage converts to fluorescent form	ROS	Ex/Em: 485/530 nm	(55)
Dihydrocalcein acetoxymethylester	Oxidation after esterase-mediated cleavage converts to fluorescent form	ROS	Ex/Em: 488/515 nm	(93, 152)
Hydroethidine (HE)	Forms a red fluorescent product that intercalates with DNA	$O_2^{-\bullet}$	Ex/Em: 518/605 nm	(19, 144, 204)
Amplex® red (10-acetyl-3,7-dihydroxyphenoxazine)	Oxidized to resorufin (colorimetric and fluorescent)	H_2O_2	Ex/Em: 563/587 nm Or Abs: 565 nm	(1, 101, 205)
FOX-2 reagent	Oxidation of iron produces a colored ferric-xylenol orange complex	H_2O_2	Abs: 560 nm	(85)
Clark's electrode	Electrochemical sensor	H_2O_2	Electrochemically	(109, 178)
Hydrocyanines	Oxidation generates extended π conjugation	$O_2^{-\bullet}$ and HO	hydro-Cy7, Ex/Em: 735/760 nm	(105)
Aryl boronic acids and esters	H_2O_2 cleaves boronate and yields fluorescent or chemiluminescent products	H_2O_2	Different generation with different Ex/Em	(43, 49, 132, 185)
Peroxalate nanoparticles containing fluorescent dyes	Oxidation of peroxalates generates high-energy dioxetanedione that excites dyes	H_2O_2	Em: 460–630 nm	(111)
Nanoreactor composed of peroxyoxalate fuel and Cy5	Oxidation of peroxyoxalate generates high-energy intermediate that excites Cy5	H_2O_2	Em: 698 nm	(115)
Hydrocyanine-conjugated, chitosan-functionalized Pluronic-based nano-carriers	Oxidation of hydrocyanine to cyanine	ROS	Ex/Em: 675/693 nm	(95)
FOSCY-1 (two linked cyanine dyes)	Oxidation of one dye releases fluorescence quenching of the other	ROS	Ex/Em: 645/668 nm and 771/813 nm	(143)

Additional examples that detect specific ROS are reviewed by Chen *et al.* (34), Lu *et al.* (120), and Freitas *et al.* (62).
[a]Wavelength may vary according to instruments.
DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′-7′-dichlorodihydrofluorescein diacetate; GRXs, glutaredoxins; roGFPs, redox-sensitive green fluorescent proteins; ROS, reactive oxygen species.

in response to changes in environmental stimuli. To this effect, mutations were incorporated into yellow fluorescent protein to introduce a pair of redox-active cysteines that create rxYFP (142). In the oxidized state, that is, on the formation of a disulfide bond between the cysteine pair, the fluorescence of rxYFP diminishes two-fold compared with the reduced state. A similar redox variant of the green fluorescent protein (roGFP1) was also independently developed and used to characterize the reducing environment of the mitochondrial matrix in HeLa cells (82). One advantage of

TABLE 3. ARYL BORONIC ESTER-BASED OXIDATION-RESPONSIVE SYSTEMS

Polymer structure	Formulation	Oxidant	Response to oxidation	Evidence of degradation	Ref.
Modified dextran	100 nm NPs	1 mM H_2O_2	Cleavage of boronic ester yields water-soluble dextran	NMR of NP, $t_{1/2}=4$ h OD of NP, $t_{1/2}=36$ min	(17)

Modified polycresol	150 nm NPs	50, 100 μM, and 1 mM H_2O_2	Degradation into small molecules on quinone methide rearrangement	NR quenching
				  $t_{1/2} \sim 45$ h at 50 μM H_2O_2
				  $t_{1/2} \sim 30$ h at 100 μM H_2O_2
				  $t_{1/2} \sim 10$ h at 1 mM H_2O_2
				Paclitaxel release
				  63% at 50 μM H_2O_2 (3 days)
				  97% at 1 mM H_2O_2 (2 days)
				  23% (2 days) in PBS only
				NMR of polymer, complete degradation within 3 days, 50 mM H_2O_2
				TEM of NP, particle deformation within 3 days, 1 mM H_2O_2
					(45)

NPs, nanoparticles, NR, Nile red; OD, optical density; PBS, phosphate-buffered saline.

the roGFP sensors compared with rxYFP is that they can be used as ratiometric sensors of redox changes, thus minimizing measurement errors (23). However, a major disadvantage of these sensors is their slow response times, which make them unsuitable for measuring dynamic physiological changes in cells (51). To overcome this issue, the human glutaredoxin (GRX1) domain was fused onto roGFP2 and obtained GRX1-roGFP2, a more sensitive probe that enables real-time monitoring of changes in redox potentials (77). Recently, GRX1-roGFP2 and a modified roGFP (roGFP2-Orp1) were employed to monitor real-time levels of oxidized GSH and H_2O_2 in various subcellular compartments of *Drosophila*, which provided insights into changes in redox environments during development and aging (2). Recently, the development of roGFP1-iL has been reported, which enables real-time measurements of redox changes in the ER (186).

HyPer. HyPer is a genetically encoded protein that specifically detects H_2O_2 levels (11). This protein consists of a circularly permuted YFP (cpYFP) that is inserted into the regulatory domain of the *E. coli* H_2O_2-sensing protein Oxy-R (11). In response to H_2O_2, the cysteines in the Oxy-R domain of HyPer form a disulfide bond, which induces a conformational change in cpYFP that shifts its excitation maximum
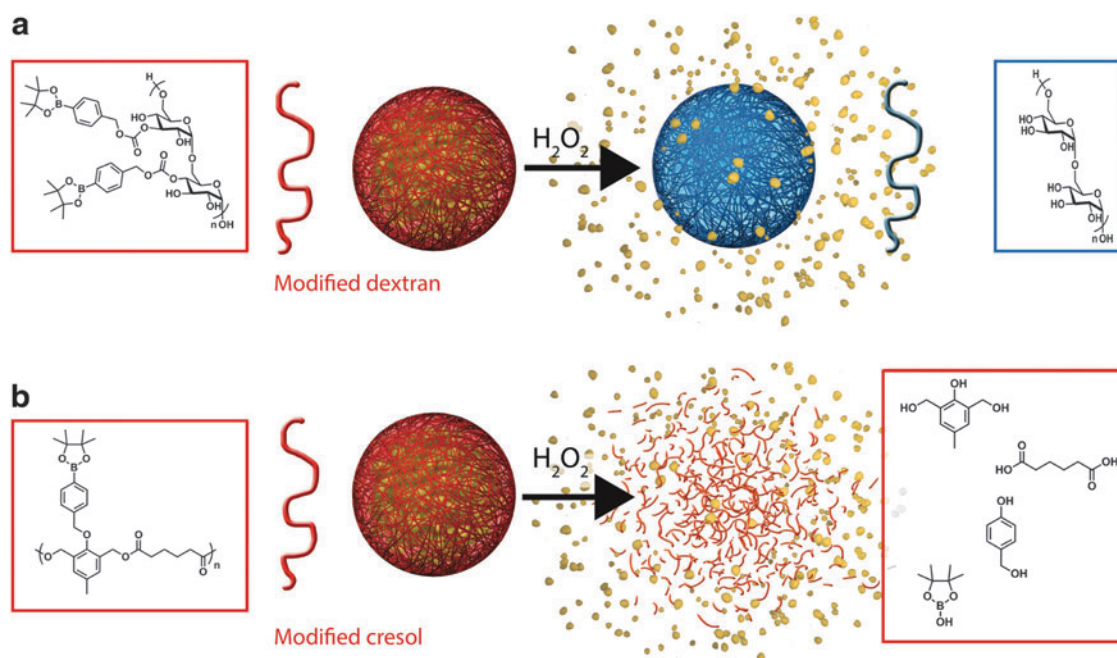

FIG. 1. **Chemical structures of aryl boronic ester-based oxidation-responsive systems and corresponding release mechanisms: modified dextran (a), modified polycresol (b)**. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

TABLE 4. EXAMPLES OF FERROCENE-BASED OXIDATION-RESPONSIVE SYSTEMS

Polymer structure	Formulation	Oxidant	Response to oxidation	Evidence of degradation	Ref.
Host pAA-βCD and guest pAA-Fc	Supra-molecular hydrogel	2.8 mM NaOCl	Oxidation of Fc to Fc$^+$ leads to inclusion complex dissociation and sol-gel phase transition Negligible K_a of Fc$^+$ and β-CD	Sol-gel transition	(138)
PVFc-b-PMMA	236 nm NCs with 25 nm PVFc patches, hexadecane core	1.3 M H$_2$O$_2$ or 1.1 mM KMnO$_4$	Swelling and hydration of PVFc nanopatches to create channels	Greater pyrene release in the presence of H$_2$O$_2$ than in KMnO$_4$ or no oxidant; normalized to greatest release (23 h)	(167)

-Fc, ferrocenyl group; -Fc$^+$, ferrocenium ion; K_a, association constant, β-CD, beta cyclodextrin; NCs, nanocapsules; pAA, poly(acrylic acid); pAA-β-CD, poly(acrylic acid) containing 4%–5% substitution of the COOH by βCDs; pAA-Fc, poly(acrylic acid) containing 2.7% substitution of the COOH by ferrocene groups; PVFc-b-PMMA, poly(vinylferrocene)-block-poly(methyl methacrylate).

from 420 to 500 nm (11). HyPer is a highly sensitive probe that can detect low concentrations of H$_2$O$_2$ in response to cellular signaling events (11). It can also be targeted to specific subcellular organelles for compartment-specific measurements of H$_2$O$_2$ levels (127).

Chemical probes and redox-sensing protein fluorophores suffer from inherent disadvantages as described earlier. The use of nanoparticles as redox sensors is a relatively new concept and has great potential for imaging redox changes in real time; we address this research direction in the perspectives section

Oxidation-Responsive Nanomaterials

In the previous sections, we provided an overview of the biological relevance of developing nanocarriers that are responsive to oxidative changes. Next, we describe the chemical systems developed so far that respond to altered oxidative microenvironments in the body. This description includes polymer structures and formulation, sensitivity level, response to oxidation mechanism, and degradation detection techniques. Oxidation sensitive systems reported in the literature can be classified into five categories by reactive groups: aryl boronic ester; ferrocenyl, proline oligomers, selenium, and thioether-responsive groups. Within these five categories, polymeric micellar aggregates (micelles, vesicles) or nanoparticles are the most common formulation. However, a few involve supramolecular hydrogels and terpolymer scaffolds. Typically, the response to oxidation includes swelling, dissolution, complex dissociation, decrosslinking, or degradation of the polymer matrix into small
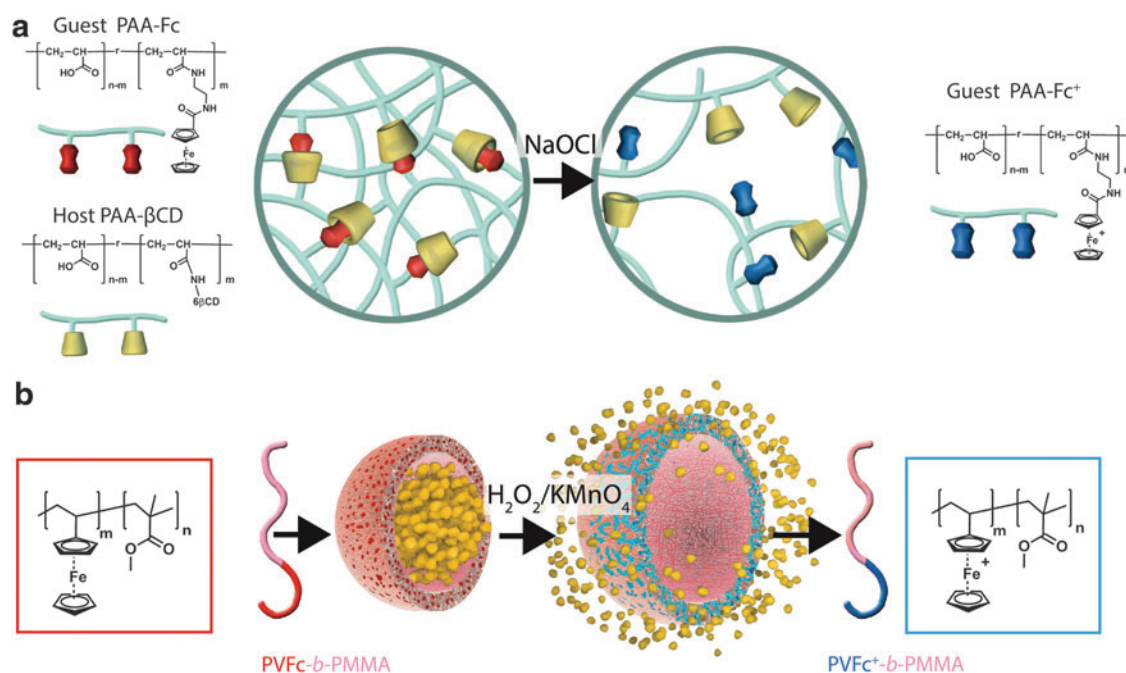

FIG. 2. **Chemical structures of ferrocene-based oxidation-responsive systems and corresponding release mechanisms: supramolecular hydrogel based on host-guest inclusion complex (a), nanocapsules containing Fc patches (b).** To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
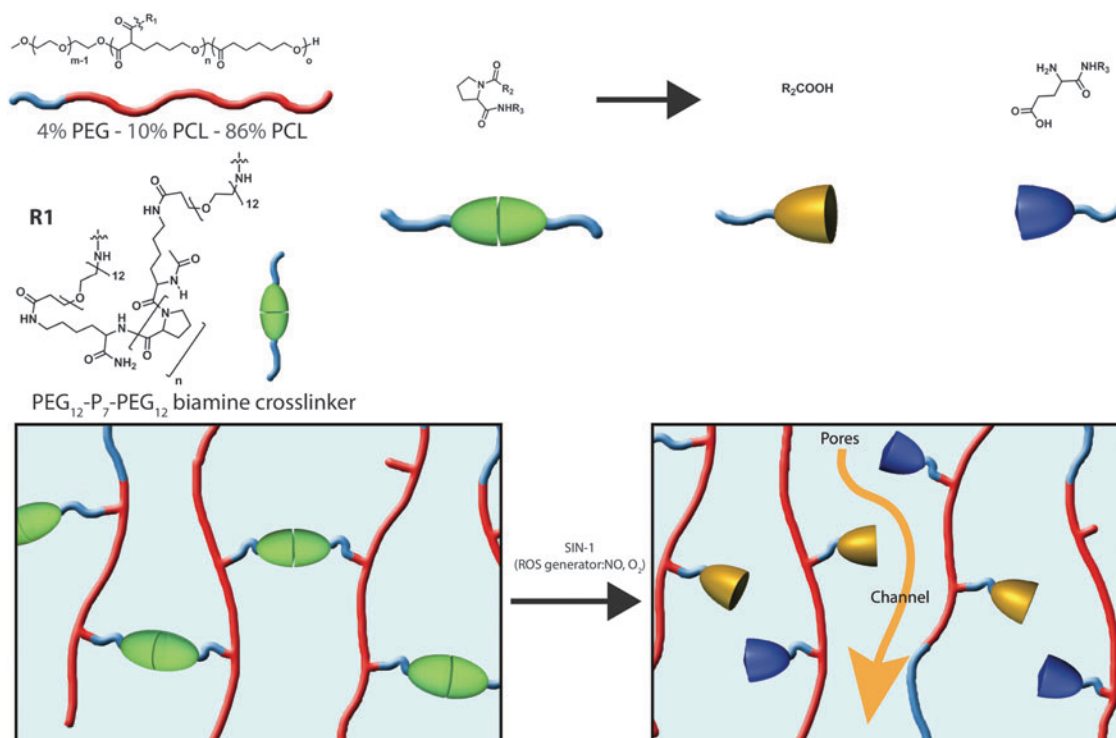
FIG. 3. **Chemical structure of proline-based oxidation-responsive system and corresponding release mechanism of a supramolecular hydrogel utilizing a PEG-oligo-proline cross-linker.** To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

molecules, all of which would enable encapsulated cargo to diffuse out.

Chemical structures that respond to oxidation

Aryl boronic esters. Recently, probes for optical H_2O_2 imaging based on aryl boronic acid and ester deprotection chemistry, which selectively and sensitively detect physiologically relevant levels of H_2O_2, have been developed (Table 2). Inspired by this breakthrough concept, nanocarriers intended to exploit redox microenvironments as triggers for drug release *in vivo* have been introduced. On oxidation, aryl boronic ester groups are oxidized to phenols that undergo quinone methide rearrangement. These ROS-reactive groups have been incorporated into polymeric systems and yield hydrophobic dextran (17) and a cresol-based

polymer (45) (Table 3). In the presence of H_2O_2, dextran particles solubilize, as the aryl boronic groups are cleaved to leave free hydroxyl groups (Fig. 1a) and the poly-cresol degrades into small molecules (Fig. 1b); if formulated into drug-carrying particles, both processes enable drug release.

Ferrocene. The oxidation of ferrocene gives a stable cation called ferrocenium, leading to a change in polarity (hydrophobic-hydrophilic switch), as the reduced form is not charged. This hydrophobic-hydrophilic transition would either enable sol-gel transition for self-healing properties (138) or enable swelling-mediated drug release applications (167) (Table 4). Thus, host-guest inclusion complexes formed by cyclodextrins and ferrocene units are no longer stable after oxidation using chemical reagents (NaOCl) or electrochemical oxidation (Fig. 2a), and nanocapsules containing patches of ferrocene (formed

TABLE 5. PROLINE OLIGOMER (P_N)-BASED OXIDATION-RESPONSIVE SYSTEMS

Polymer structure	Formulation	Oxidant	Response to oxidation	Evidence of degradation	Ref.
4% PEG-86% PCL-10% cPCL crosslinked with PEG$_{12}$-P$_7$-PEG$_{12}$	Terpolymer scaffolds	1 mM SIN-1, which decomposes into NO and O$_2^-$ (28 days)	Creation of pores in the polymeric scaffold on oxidative cleavage of proline residues (de-crosslinking)	13.5% greater mass loss than in scaffolds cross-linked with PEG-dihydrazide DSC, ~5% lower heat capacity for melting point transition attributable to ROS-mediated hydrolysis	(202)

cPCL, poly(carboxy-ε-caprolactone); DSC, differential scanning calorimetry; percentages indicate molar proportions; PEG, poly(ethylene glycol); PCL, poly(ε-caprolactone); SIN-1, morpholinosydnonimine.
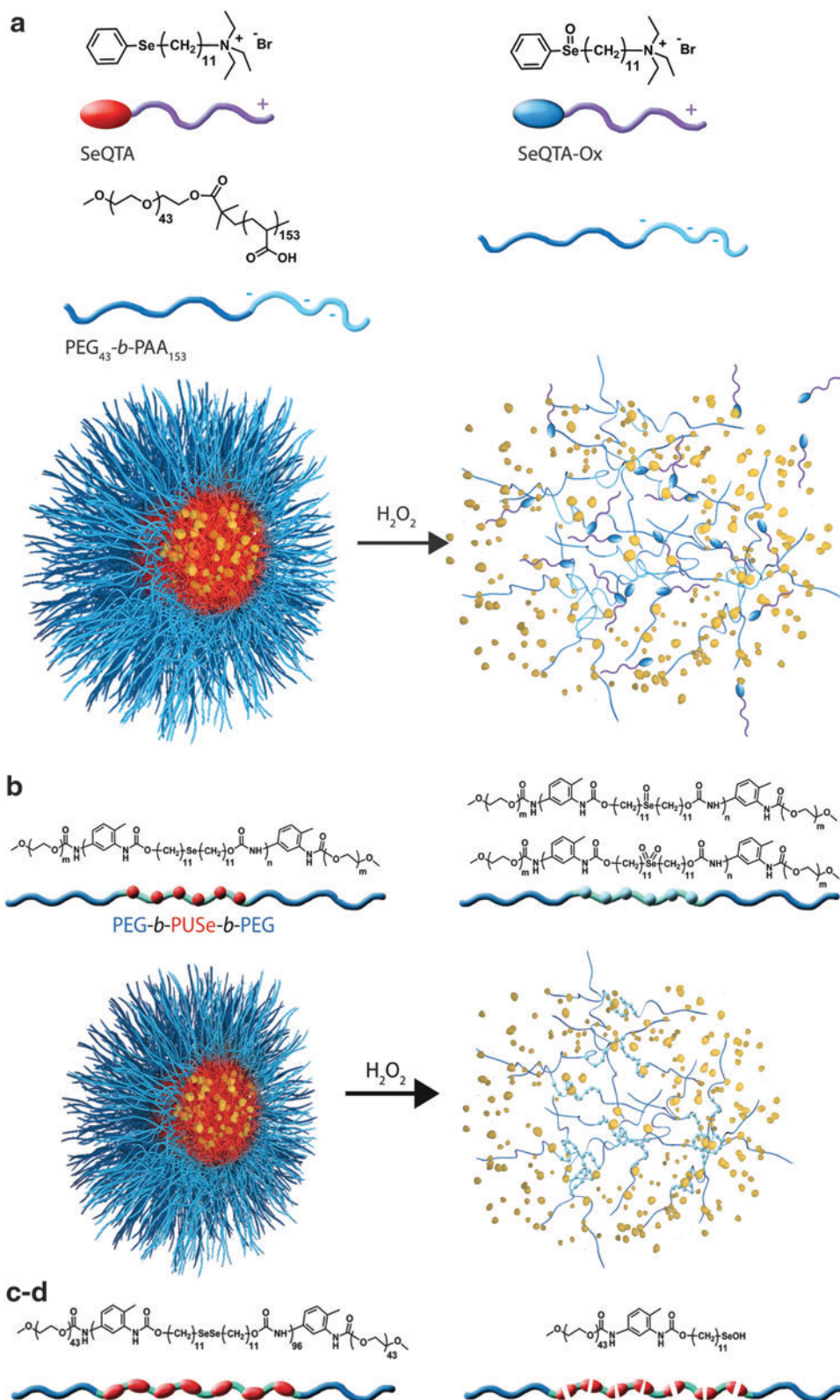
FIG. 4. **Chemical structure of selenium-based oxidation-responsive systems and corresponding release mechanisms: noncovalently connected polymeric superamphiphiles (a), main chain monoselenide and diselenide polymers (b–d), and side-chain selenium-containing polymer (e).** To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
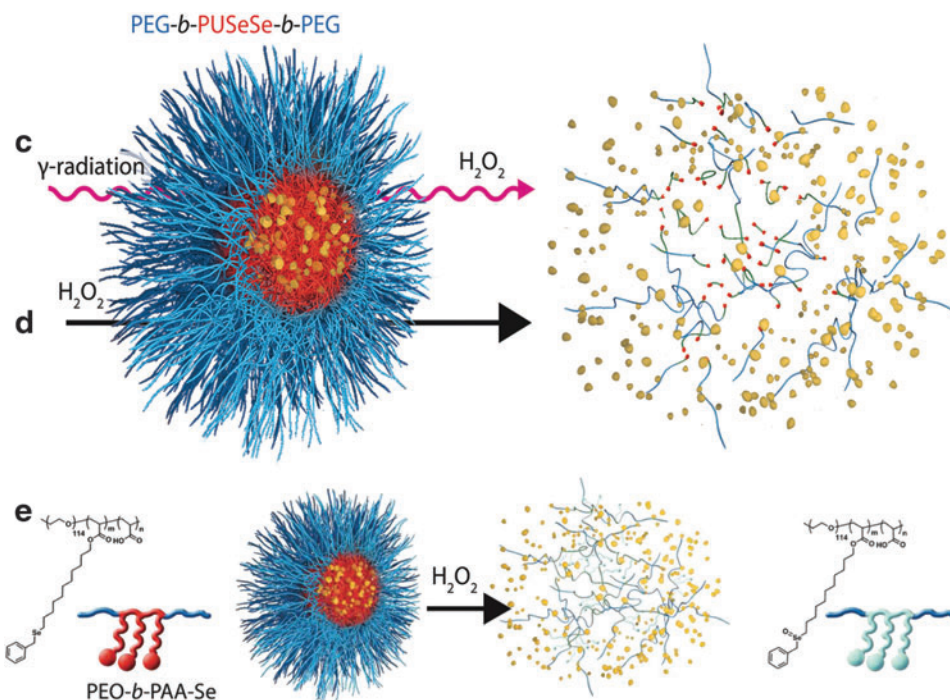
FIG. 4. (*Continued*).

spontaneously on formulation of polyvinyl ferrocene-co-poly (methyl methacrylate) release their payload on oxidation (H_2O_2, $KMnO_4$) through swollen hydrophilic channels created within the containers (Fig. 2b). Interestingly, while ferrocene itself is benign, its oxidized ferrocenium cationic analog has been shown to be toxic to tumoral cells (antiproliferative activity). Indeed, with an unpaired electron in one of the nonbonding orbitals, it becomes a stable free radical species involved in a redox-mediated toxic mechanism through hydroxyl radical formation in physiological environments (68). Other examples of ferrocene-based designs have been recently reported in the literature. They rely on electrochemical oxidation [liposome (39)], host-guest interaction-based vesicle (200), and gel (104) or chemical oxidation by large amounts of iron oxide in a micellar assembly (88). In this review, we focused on carrier designs with the potential for application in ROS-triggered drug delivery. These systems will not be a part of our classification because they do not respond to relevant biological clues.

Proline oligomers. Free amino acids and amino acids in proteins, especially histidine, proline, arginine, and lysine, are susceptible to ROS-mediated and metal-catalyzed oxidation (4, 166). On oxidative cleavage of peptide bonds, the generation of carbonyl derivatives may occur by many different mechanisms. The incorporation of proline oligomer (Pn) into poly(ethylene glycol) (PEG) cross-linkers (PEG-Pn-PEG) within a nonresponsive polymeric scaffold (Fig. 3 and Table 5) yields a system that becomes porous on oxidation-induced cleavage of the prolines, leading to gradual network hydrolysis (202).

Selenium-containing amphiphilic block copolymers. Selenium-containing amphiphilic block copolymers, including noncovalently connected polymeric superamphiphiles (81) (Fig. 4a), and polymers containing a monoselenide (121)

(Fig. 4b), diselenide in the main chain (122, 123) (Fig. 4c, d) or selenium in the side chain (150) (Fig. 4e), have also been investigated as potential ROS-responsive systems (123) (Table 6 and Fig. 4c). In each case, the amphiphilicity of the block copolymer causes self-assembly into spherical micellar aggregates. On oxidation, selenide groups are converted into selenoxide and selenone (monoselenide) or seleninic acid (R-Se-OH, in the case of diselenide bonds), which reduces amphiphilicity or cleaves the polymer, causing the aggregates to fall apart. The oxidation of selenium in side chains of amphiphilic block copolymers can also be reversed to selenide under reduction with vitamin C (150).

Thioethers. So far, thioether-based oxidation-responsive systems are the most investigated (Table 7). On oxidation, thioether moieties are converted into sulfones and sulfoxides that create highly hydrophilic molecules due to the strong dipole between oxygen and sulfur. At the molecular level, the increase in polarity and water affinity (solvation) leads to morphological disruption (76, 139) (Fig. 5a, b) or swelling and solubilization (3, 149) (Fig. 5c, d). Thioketal linkages present in all polymer subunits are completely cleaved in the presence of KO_2 (196) (Fig. 5e) or by the intracellular ROS present in cancer cells (162). Finally, in a dual-response strategy, thioether oxidation accelerates the cleavage of hydrolytically sensitive ketal groups by enabling greater solvation of the polymer (125) (Fig. 5f).

Silicon oxidation enhanced by the addition of an oxidant. Another example showed that oxidation-triggered hydrolysis of mesoporous silicon microparticles in the presence of peroxynitrite generated *in situ* by 1–2 mM morpholinosydnonimine (SIN-1) could be a potential efficient platform for ROS-triggered long-term release of covalently attached anticancer drug doxorubicin (198). However, this

TABLE 6. EXAMPLES OF SELENIUM-BASED OXIDATION-RESPONSIVE SYSTEMS

Polymer structure	Formulation	Oxidant	Response to oxidation	Evidence of degradation	Ref.
SeQTAPEG$_{43}$-b-PAA$_{153}$	Polymeric superamphi-phile micelles	0.1% H_2O_2 ($\sim$30 mM)	Disassembly of micelles after oxidation of selenide groups into hydrophilic selenoxide	TEM, micelles disappeared in 1 h Release of FluNa over $\sim$11 h, control not shown	(81)
PEG-b-PUSe-b-PEG	71 nm block copolymer aggregates	0.1% H_2O_2 ($\sim$30 mM)	Dissociation due to oxidation of selenides into hydrophilic selenoxide or selenone groups	TEM, aggregates disappear in 5 h XPS, selenoxide, and selenone formation Greater dox release (10 h) than from polysulfide analogs (72% vs. 42%)	(121)
PEG-b-PUSeSe-b-PEG	67 nm micellar aggregates	γ radiation (5 and 50 Gy)	Oxidation of diselenide groups into seleninic acid by the oxidative species (HO$^{\bullet}$, $^{\bullet}$HO$_2$, and H_2O_2) generated by water during γ-radiation Diselenide bonds cleavage induces partial disassembly and drug release	FTIR, appearance of seleninide acid TEM, swollen micelles (5 Gy) Collapsed irregular aggregates (50 Gy) Dox release in 8 h, 72% (50 Gy) 43% (5 Gy) 0% (0 Gy)	(123)
PEG-b-PUSeSe-b-PEG	76 nm micellar aggregates	0.1% H_2O_2 ($\sim$30 mM) 0.01% H_2O_2 ($\sim$3 mM)	Se-Se bonds are cleaved and oxidized to seleninic acid Diselenide bonds cleavage induces complete disassembly and drug release	NMR, appearance of seleninide acid TEM, micelle disassembly in 5 h in 0.1% H_2O_2 RB release within 5 h in 0.01 and 0.1% H_2O_2; no release in absence of H_2O_2	(122)
PEO-b-PAA-Se-64[a]	33 nm micellar aggregates	0.1% H_2O_2 ($\sim$30 mM)	Structural dissociation due to the oxidation of side-chain selenide groups into hydrophilic selenoxide groups	TEM, disassembly of the micelles in 8 h NMR and FTIR of a model compound; selenoxide appearance NR quenching within 25 h; control not shown	(150)

[a]Grafting ratio.

SeQTA, selenium-containing surfactant; XPS, X-ray photoelectron spectroscopy; FluNa, fluorescein sodium; Dox, doxorubicin, FTIR, Fourier transform infrared spectroscopy; RB, Rhodamine B.

approach requires a modification of the drug from its normal form, which might limits its application.

Formulation of oxidation-responsive materials

Heavy reliance on the formulation of ROS-sensitive polymers into micellar aggregates may inherently limit the translation of these studies into animal models. While these self-assembled amphiphilic block copolymers have several potential advantages compared with other types of nanosized drug carriers, including an excellent biocompatibility, controllable size in a range that can escape the vasculature (20–100 nm), efficient loading of hydrophobic drugs, and prolonged circulation time provided by their layer of hydrophilic antifouling shell (stealth effect) (47), these may be outweighed by their disadvantages. Specifically, after an intravenous injection, dilution causes micelles and vesicles to dissociate and aggregate, nonspecifically releasing drugs,

which decreases their targeting capability. Further, these assemblies do not efficiently penetrate tumors and are inefficiently taken up by cells due to hydrophilic surface shielding. Thus, despite the fact that polymeric micelles may enable faster release than nanoparticles on triggered destabilization, as dissociation time is faster than polymer degradation, their apparent sensitivity *in vitro* may not be a true advantage. For this reason, hydrophobic nanoparticles composed of polymers that react with ROS may be more promising candidates, offering both better physicochemical stability and more reliable tumor permeation.

Challenges in evaluating in vivo *applicability*

Unfortunately, it is difficult to compare the efficiency of release in response to ROS both across and within categories. The studies reviewed here employed varying oxidants (H_2O_2, O_2, NaOCl, KMnO$_4$, NO, and O_2^- produced by SIN-1, H_2O_2,

TABLE 7. EXAMPLES OF THIOETHER-BASED OXIDATION-RESPONSIVE SYSTEMS

Polymer structure	Formulation	Oxidant	Response to oxidation	Evidence of degradation	Ref.
EG_{16}-PS_{50}-EG_{16}	Triblock macro-amphiphiles unilamellar vesicles; membrane ~ 8 nm thick	0.03%–10% H_2O_2 (~ 9 mM-3 M)	HLB alteration and vesicle disruption due to the oxidation of the hydrophobic PPS blocks into hydrophilic polypropylene sulfoxides and sulfones	OD, clear: 10% H_2O_2 (10 h), 0.03% (300 h) TEM, vesicle number, shape change: 10% H_2O_2 (few min), 0.03% H_2O_2 (150 min) NMR, appearance of sulfoxides and sulfones	(139)
Poly(PS_{74}-b-DMA_{310})	100 nm micelles	1.1 mM-1.1 M H_2O_2, 1–100 μM $ONOO^-$, or 1–100 mM SIN-1	PPS oxidation causes micelles disassembly into unimeric polymers and cargo release	NMR, sulfone formation in H_2O_2 DLS + TEM, disruption, 1.1 M H_2O_2 NR or DiO release correlating to H_2O_2, $ONOO^-$, or SIN-1 conc.	(76)
Disulfide cross-linked PPS	25–225 nm Pluronic F127-stabilized PPS NP	5%–10% H_2O_2 (~ 1.5–3 M)	Swelling and solubilization after conversion to hydrophilic polypropylene sulfoxides and sulfones	OD, loss of turbidity: 5% H_2O_2 (30 h), 10% H_2O_2 (95 h)	(149)
PPS	150 nm Pluronic F127-stabilized PPS NP	25–200 ppm NaOCl CPO and hMPO + 200 mM NaCl, 500 μM H_2O_2	PPS oxidation causes swelling and cargo release	OD, transmittance incr. Reichardt's dye release, 98%, 5 min (2000 ppm NaOCl) NR: transmittance incr. 17%, fluor. decr. 70% (CPO); transm. incr. 10%, fluor. decr. 80% (hMPO)	(3)
PPADT	600 nm NP	KO_2 (unknown level)	Cleavage of thioketal linkages on oxidation	GPC, PPADT degradation in 8 h (high or low pH, no change)	(196)
Poly(thio-ether ketal)	200 nm NP	100 mM H_2O_2 pH: 5, 6.5, and 7.4	Dual response: thioether oxidation into sulfoxides produces more swollen and hydrophilic particles, enabling ketal hydrolysis and payload release	DLS, NP degradation, pH 5/100 mM H_2O_2, 15 h NMR, complete hydrolysis in 2 d NR quenching: >80% in 25 h, 100 mM H_2O_2 (pH 5 or pH 7); <30% in abs. OVA release: ~ 100% in 100 mM H_2O_2 (pH 6.5); <20% without H_2O_2	(125)

DLS, dynamic light scattering; EG, ethylene glycol; GPC, gel permeation chromatography; HLB, hydrophilic to lipophilic balance; hMPO, human myeloperoxidase; Mn, number average molecular weight; OD, optical density; PPS, poly(propylene sulfide); PS, propylene sulfide; PGSE, pulse gradient spin echo; PPADT, poly-(1,4-phenyleneacetone dimethylene thioketal); poly(PS_{74}-b-DMA_{310}), a diblock polymer of propylene sulfide (PS) and N,N-dimethylacrylamide; DiO, (3,3′-dioctadecyloxacarbocyanine perchlorate); CPO, chloroperoxidase; OVA, ovalbumin Alexa Fluor 594; NMR, nuclear magnetic resonance.
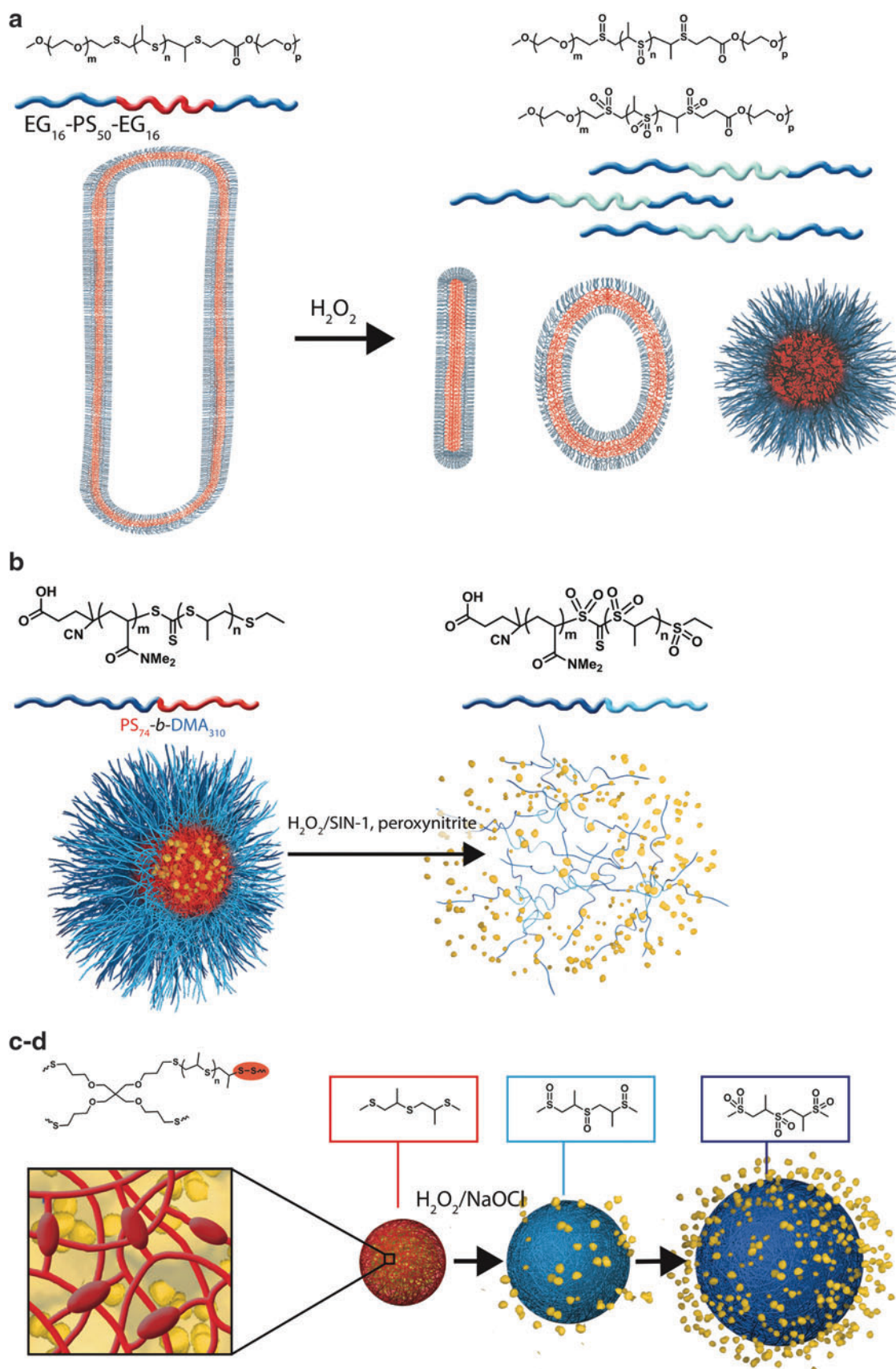
FIG. 5. Chemical structure of thioether-based oxidation-responsive systems and corresponding release mechanisms: thioether (a–d), thioketal (e), and thioether-ketal (f). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars
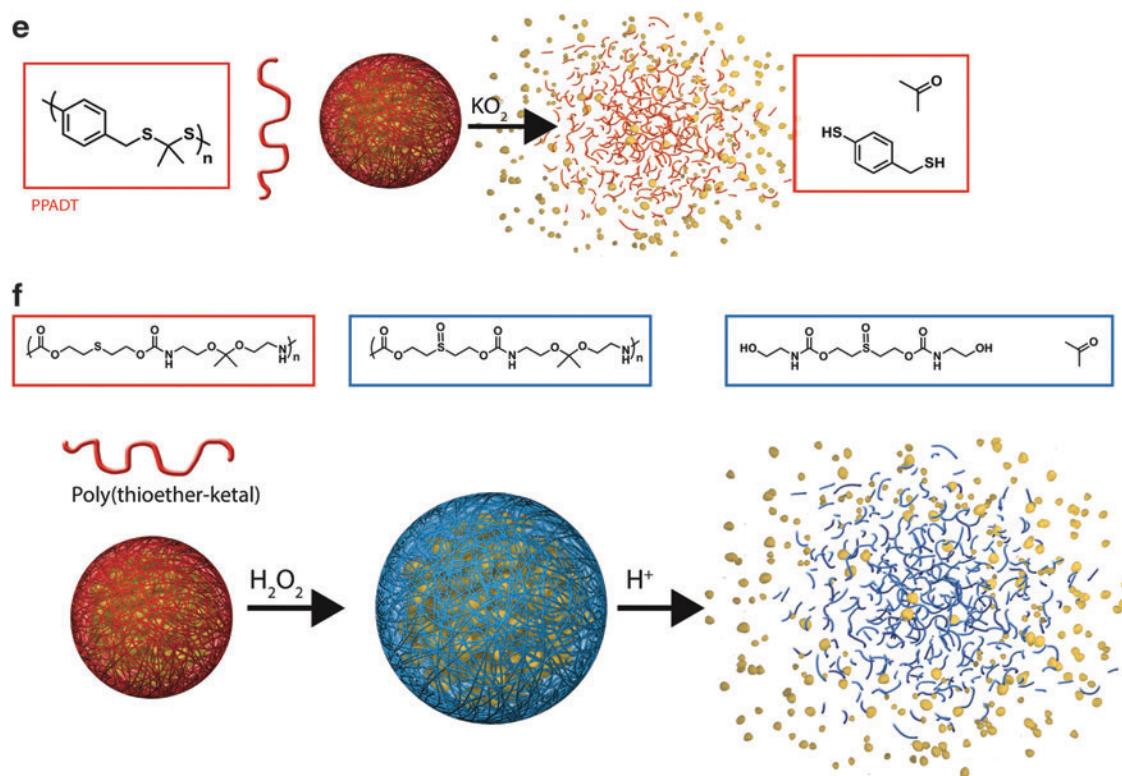
FIG. 5. (*Continued*).

and radicals generated by γ-radiation, KO$_2$), concentrations of ROS (for H$_2$O$_2$, from 50–100 μM to concentrations as high as 3 M), and ratios between the responsive polymer assembly and oxidative agent. While some papers claim that 0.1% ($\sim$30 mM) H$_2$O$_2$ is a quite mild oxidative environment, this level is far from the biologically relevant range (50–100 μM) (80). However, even polymeric assemblies that release cargo only in response to high concentrations of H$_2$O$_2$ *in vitro* may be sensitive enough, given that H$_2$O$_2$ is converted to hydroxyl radical in the body spontaneously on an interaction with transition metal ions, such as iron, and hydroxyl radical is more reactive than H$_2$O$_2$. Another important consideration is that oxidizing agents can affect pH, so the apparent ROS sensitivity of chemical structures that degrade or change hydrophilicity in response to pH may be inaccurate. Molar concentrations of H$_2$O$_2$ in water can cause the pH to be acidic, while molar concentrations of NaOCl can shift it above 9.

Moreover, within the same study, different detection methods yield different results (transmission electron microscopy (TEM), payload release, nuclear magnetic resonance spectroscopy, and light scattering), which further limits any comparison. More precisely, detection methods vary in their sensitivity; for example, turbidity measurements are far less sensitive than fluorescence, and Nile red quenching may or may not correlate with degradation, as water diffusion into the hydrophobic core would also cause quenching. Calculating payload release relative to the total amount encapsulated and not only as a function of time would give an evaluation of release that could more easily be compared across various nanocarrier assemblies and cargos.

Thus, additional measures of nanocarrier degradation such as TEM or real quantification of drugs by liquid chromatography-mass spectroscopy should become standard within the field. Indirect methods using light scattering or transmission do not provide as detailed an understanding of the mechanism by which particles degrade as does TEM imaging. On degradation, nanoparticle polydispersity increases, and the refractive index and viscosity of the solution change. Since dynamic light scattering calculations assume constant refractive index and viscosity values, these readings can be unreliable. Further, TEM enables a more detailed understanding of how particles degrade and release cargo, as changes in morphology can be directly visualized. For instance, our group used TEM to detail significant changes in nanoparticle morphology after treatment with H$_2$O$_2$, such as ripping or crumpling of the structures, as well as particle expansion (45).

A few direct comparisons of polymeric carriers' sensitivity to oxidative agents have been made. Only one study has compared the sensitivity of polyselenide block copolymer-based micellar aggregates with polysulfide analogues and reports that selenide moieties are more sensitive: The lower binding energy of Se-C bond compared with S-C bond makes Se-containing molecules more sensitive to oxidative stimuli (121).

The design of some systems to respond to a combination of acidic pH with oxidative conditions (125) may enhance their usefulness in specific disease conditions. Since the pH in tumors and inflamed tissues is known to be slightly acidic, nanoparticles that degrade specifically in response to both stimuli may better target drug delivery to those conditions.

In diselenide cross-linked polymeric carriers (122), the linkages are also cleaved by a reduction in the presence of thiols. For example, in the dual-responsive assemblies
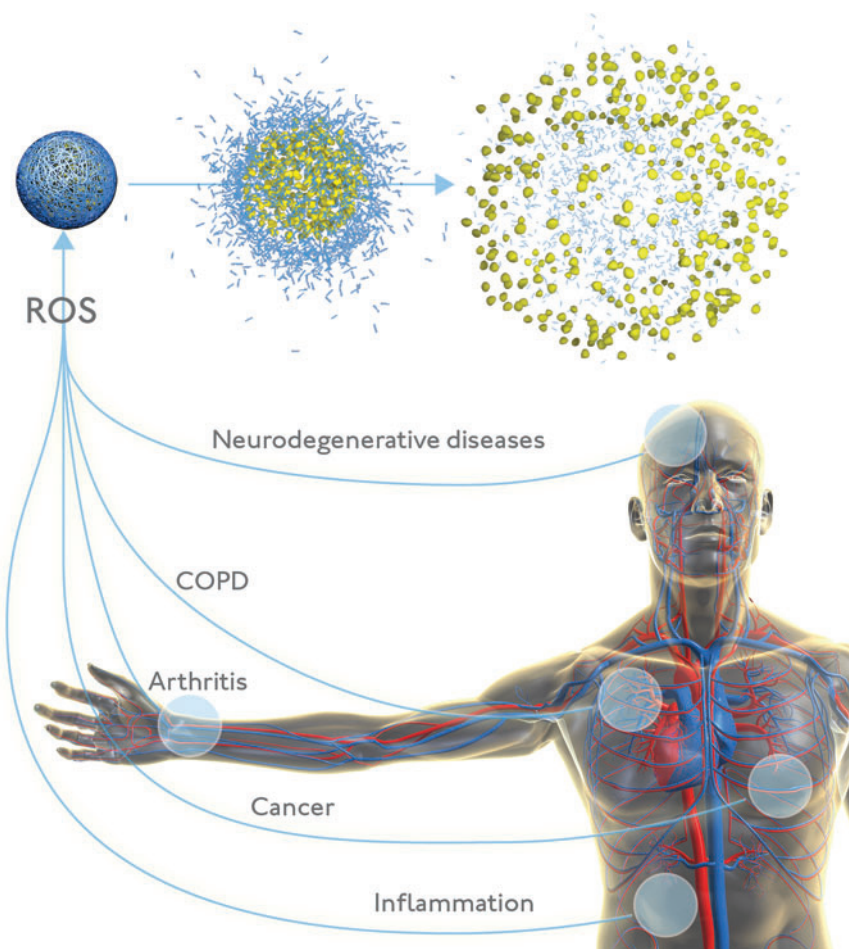
FIG. 6. Reactive oxygen species-responsive nanocarriers that release payload in response to redox changes in the microenvironment have tremendous potential as diagnostic and therapeutic agents for a variety of pathophysiological conditions. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(diselenide block copolymers), *in vitro* release was demonstrated in the presence of both oxidizing (H_2O_2) and reducing agents (GSH) (122). Since the cytosol of healthy cells has a strong reducing environment, such particles could release cargo through the reducing mechanism if endocytosed. However, the intracellular accumulation of polymeric nanoparticles by nonendothelial cells is uncommon; significant uptake generally requires that particles be decorated with targeting moieties or cell-penetrating peptides [reviewed in (206)]. Circulating nontargeted particles are more likely to be either phagocytosed or encounter an extracellular environment that is oxidative (especially in the diseases described earlier), triggering release.

Biologically relevant ROS-responsive systems. This review highlights various chemistries that can be used to formulate drug carriers which are sensitive to oxidation. In most studies developing novel ROS-sensitive nanocarriers, the assessment of payload release is performed *in vitro* using concentrations of oxidants that are several fold higher than biologically relevant levels. While a huge challenge in testing oxidation-responsive carriers is the extremely short half life of these oxidant molecules (ROS/RNS), model cell culture systems can be considered useful tools for providing insights into the sensitivity of such nanocarriers. For example, macrophage (*e.g.*, Raw264.7) and neutrophil (*e.g.*, mouse promyelocyte) cell lines can be manipulated to produce an oxidative burst.

Studies in activated macrophages and neutrophils have demonstrated that several systems, including a boronic ester-modified polymeric particle (45), a polymer-based hydrogel cross-linked with proline oligomers (202), and micelles composed of a poly(propylene sulfide) (PPS) core (76), respond to biological levels of H_2O_2. The first of these studies was especially well controlled, as it compared responsiveness of the designed ROS-sensitive polymer with that of poly(lactic-*co*-glycolic) acid (PLGA) and a control polymer lacking ROS-reactive groups; neither control releases cargo in response to activated neutrophils (45). This finding may lead to the polymer's use in targeting drugs to tissues that are characterized by a high level of neutrophils (*e.g.*, inflammatory diseases such as SIRS and COPD). Though the PPS micelle study only demonstrated a 10% difference between release induced by activated and nonactivated macrophages, their release assay was creative: a reduction in fluorescence resonance energy transfer between co-encapsulated dyes (76). Systems such as this that were demonstrated to respond to activated macrophages could prove useful for targeting drugs to sites which are rich in these cells, such as vulnerable atherosclerotic plaques. Since ROS-responsive systems may prove useful for the delivery of vaccine formulations, one group has tested their ability to enhance CD8^{+} presentation by T cells on uptake by dendritic cells *in vitro*. Broaders *et al.* showed that their aryl boronic ester-modified dextran particles produced a 27-fold greater enhancement of CD8^{+} presentation than ovalbumin in PLGA (17). Another interesting application of ROS-responsive polymers is for gene delivery to cancer cells with high ROS/RNS. ROS/RNS-sensitive

poly(amino thioketal) was shown to have a higher efficiency of gene delivery and expression compared with non-degradable poly-amine or the commonly used transfection agent (branched polyethyleneimine) (162). In addition, the authors conjugated a GRP78 binding peptide to the polymer to further enhance uptake by cancer cells.

The only *in vivo* investigation on oxidation-responsive nanoparticles to date shows the advantage of anti-inflammatory agents in oxidation-sensitive particles compared with a slowly degrading material, PLGA, for oral delivery. In a murine model of ulcerative colitis induced by the administration of dextran sodium sulfate in drinking water (196), TNFα-siRNA complexed with DOTAP (cationic lipid, 1,2-dioleoyl-3-trimethyl-lammonium-propane) encapsulated into thioketal particles (TNFα-TKNs) induced a ten-fold decrease in TNFα-mRNA expression in the colon; while TNFα-siRNA in PLGA had no significant effect. This superiority of thioketal particles was likely due to the combined effect of the stability of TKNs against the harsh gastric environment and their ability to degrade in response to ROS produced by inflamed intestinal tissue.

Though only one ROS-responsive system has been tested *in vivo*, others have demonstrated the utility of nanoparticles for delivering reactive antioxidants. For example, a novel redox nanoparticle (RNP^O) consisting of a self-assembling ampiphilic block copolymer containing a derivative of the antioxidant TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl) in the hydrophobic segment has been shown to be more effective than free TEMPOL or mesalamine (an anti-inflammatory compound) at treating colitis in mice (190).The confinement of the nitroxide radical-containing TEMPOL derivative in the hydrophobic core of RNP^O nanoparticles enhanced its biocompatibility, and the nanoparticles' size (40 nm) caused them to accumulate in the colonic mucosa. A recent report described the antioxidant properties of nanoparticles (PVAX) formulated from copolyoxylate containing vanillyl alcohol (VA) (110). The PVAX polymer consists of peroxalate ester bonds along with VA in the backbone. Under physiological conditions PVAX is hydrolytically cleaved and releases VA, which has therapeutic effects (anti-inflammatory, antioxidant, and anti-cociceptive) (110). In addition, the PVAX nanoparticles were shown to scavenge hydrogen peroxide in a concentration-dependent manner. Finally, the authors demonstrated the therapeutic efficacy of these nanoparticles in an ischemia/repurfusion model in mice (110). These examples further illustrate that nanoparticles can be designed as therapeutics rather than simply as carriers.

Perspectives

The future of these ROS/RNS-responsive carriers depends on whether they are truly sensitive to the levels of ROS/RNS produced in disease conditions. Given the scarcity of studies that have examined this directly, predicting which chemistries will prove most useful remains impossible. Thus, extensive further studies are needed for examining the effectiveness of those ROS/RNS-responsive chemistries that prove sensitive to biologically produced ROS/RNS in cell culture systems. These studies should be judicious in their choice of disease model, as each system likely has varying sensitivity to ROS, and dosing route, as nontargeted particles may not accumulate in intended tissues if administered systemically. Further, while the exact ROS levels and species in various disease models remain unknown (see section on Detection of ROS/RNS in biological

systems), the variety of mechanisms of ROS/RNS production suggests that some disease states likely involve much higher levels than others. The disease models described thus far to test redox-responsive nanoparticles rely on the production of an aggravated immune response (*e.g.*, dextran sodium sulfate-induced colitis), which likely produces a tremendous amount of ROS/RNS due to oxidative burst from accumulated phagocytes. While such a model has direct applicability in terms of human disease (ulcerative colitis), it would be interesting to determine whether some ROS/RNS-responsive carriers respond as well to oxidative stress produced in other conditions (*e.g.*, cancer).

An especially exciting potential application of oxidation-responsive nanoparticles would be to directly assess the question of ROS levels in various disease states (Fig. 6), filling in the unknown that currently limits the assessment of *in vivo* applicability. The applicability of existing chemical ROS probes is largely restricted to cell culture, as they have poor retention in animal models. Genetically encoded proteins, on the other hand, need to be stably incorporated into the genome or transfected into the tissue being studied. While these protein ROS sensors are useful tools for cell and animal research, sensors that enable the measurement of ROS in humans would be even more useful. ROS-responsive nanoparticles may represent this translatable technology, as they are generally biocompatible, circulate for extended periods, and may be targeted to specific tissues.

There is a growing body of literature on nanoparticle-based ROS/RNS sensors that incorporate various imaging modalities (fluorescence, luminescence, Raman scattering, *etc.*) to detect redox changes, and some of these have been used to image ROS/RNS *in vivo* in tumors [reviewed in detail elsewhere (183)]. Those applied *in vivo* have so far been limited to chemiluminescent sensors, which detect the ROS produced in response to lipopolysaccharide-induced inflammation (111, 115). However, neither of these studies attempted to quantify ROS levels, suggesting that chemiluminescence does not correlate with ROS concentration. Thus, nanosystems that provide concentration-dependent signals may be more promising for this purpose; sensors based on the quenching of metallic nanoparticle fluorescence show such a response *in vitro*. For example, CdSe/ZnS quantum dots conjugated to oxidized cytochrome c; oxidation by $O_2^{\bullet-}$ enhances fluorescence (113). Further along these lines, oxidation-responsive nanoparticles could also be employed to develop an ROS-detecting magnetic resonance imaging contrast system *via* release of Gd-based contrast agents from hydrophobic assemblies and resulting in de-quenching of contrast enhancement (188). In summary, the oxidation-sensitive chemistries described here offer exciting new prospects in the field of nanomedicine, specifically targeting microenvironments with perturbations in redox levels.

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Abbreviations Used

β-CD = beta cyclodextrin
ALS = amyotrophic lateral sclerosis
Bcr-Abl = Breakpoint cluster region- Abelson murine leukemia
CD8$^+$ = cluster of differentiation 8 positive
CoA = coenzyme A
COPD = chronic obstructive pulmonary disease
cPCL = poly(carboxy-ε-caprolactone)
CPO = chloroperoxidase
cpYFP = circularly permuted yellow fluorescent protein
DCFH-DA = 2'-7'-dichlorodihydrofluorescein diacetate
DiO = (3,3'-dioctadecyloxacarbocyanine perchlorate)
DLS = dynamic light scattering
DMA = N,N-dimethylacrylamide
DNA = deoxyribonucleic acid
DOTAP = 1,2-dioleoyl-3-trimethylammonium-propane
Dox = doxorubicin
DSC = differential scanning calorimetry
ECM = extracellular matrix
EC-SOD = extracellular superoxide dismutase
EG = ethylene glycol

ER = endoplasmic reticulum
ETC = electron transport chain
Fc = ferrocenyl group
Fc$^+$ = ferrocenium ion
FluNa = fluorescein sodium
FTIR = Fourier transform infrared spectroscopy
GFP = green fluorescent protein
GPC = gel permeation chromatography
GPX = glutathione peroxidase
GRP78 = glucose-regulated protein 78
GRX = glutaredoxin
GSH = glutathione
H$_2$DCFDA = dichlorodihydrofluorescein diacetate
HE = hydroethidine
HLB = hydrophilic to lipophilic balance
hMPO = human myeloperoxidase
IFNγ = interferon gamma
IL-1 = interleukin-1
K_a = association constant
Mn = number average molecular weight
MMP = matrix metalloproteinase
MPO = myeloperoxidase
MPRO = mouse promyelocyte
mRNA = messenger ribonucleic acid
mt DNA = mitochondrial DNA
mtNOS = mitochondrial nitric oxide synthase
NADPH = nicotinamide adenine dinucleotide phosphate
NC = nanocapsule
NMR = nuclear magnetic resonance
NOS = nitric oxide synthase
NOX = nicotinamide adenine dinucleotide phos-phate oxidase
NP = nanoparticle
NR = Nile red
OD = optical density
OVA = ovalbumin Alexa Fluor 594
pAA = poly(acrylic acid)
pAA-β-CD = poly(acrylic acid) containing 4%–5% substitution of the COOH by beta cyclodextrins
pAA-Fc = poly(acrylic acid) containing 2.7% substitution of the COOH by ferrocene groups
PBS = phosphate-buffered saline
PCL = poly(ε-caprolactone)
PD = Parkinson's disease
PEG = poly(ethylene glycol)
PGSE = pulse gradient spin echo
PINK1 = phosphatase and tensin homolog-induced putative kinase 1
PLGA = poly(lactic-*co*-glycolic) acid
PMNs = polymorphonuclear leukocytes
Pn = proline oligomer
PPADT = poly-(1,4-phenyleneacetone dimethylene thioketal)
PPS = poly(propylene sulfide)
PS = propylene sulfide
PVAX = copolyoxylate containing vanillyl alcohol
PVFc = poly(vinylferrocene)
PVFc-*b*-PMMA = poly(vinylferrocene)-block-poly(methyl methacrylate)

Abbreviations Used (Cont.)

RA = rheumatoid arthritis
RB = rhodamine B
ROS = reactive oxygen species
RNP^O = redox nanoparticle
RNS = reactive nitrogen species
roGFP = redox-sensitive green fluorescent protein
rxYFP = redox-sensitive yellow fluorescent protein
SeQTA = selenium-containing surfactant
SIN-1 = morpholinosydnonimine
siRNA = small interfering ribonucleic acid

SIRS = systemic inflammatory response syndrome
SOD = superoxide dismutase
T_H17 cells = autoantigen-sensitized T cells
TEM = transmission electron microscopy
TEMPOL = 4-hydroxy-2,2,6,6-tetramethylpiperidin-1-oxyl
TKN = thioketal nanoparticle
TNFα = tumor necrosis factor alpha
VA = vanillyl alcohol
XPS = X-ray photoelectron spectroscopy
YFP = yellow fluorescent protein