Implications of poly(alkyl cyanoacrylate) nanoparticle-induced cellular stress responses with focus on autophagy

Ph.D. Thesis

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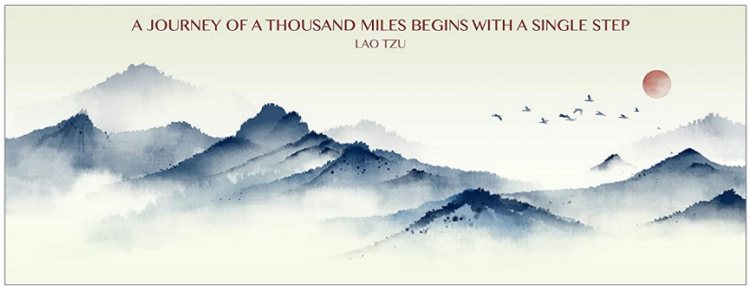

Det er i motbakke det går oppover (Rune Gokstad)

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SUMMARY

Nanoparticles (NPs) are exploited in the development of drug delivery systems to increase bioavailability and drug targeting, minimizing side effects for the patient. Inherent to their size, NPs are positioned between bulk structures and atomic or molecular structures, acquiring unique properties compared to their bulk counterparts. Upon interaction with living cells the altered properties of NPs may lead to undesired and unforeseen physiological consequences. To close the gap between the development of novel medicines exploiting NPs and clinical translation, we need to decipher the consequences of their interactions with biological systems. The NP physicochemical characteristics, such as size, shape, surface charge, and coating, play key roles in determining their biocompatibility. In biological fluids proteins adsorb to the NP surface forming a protein corona around the NP, defining the biological identity of the NP seen by the functional machinery of cells. To maintain proper function and integrity, cells need to adapt and respond to their continuously changing environment, such as treatment with NPs. In this thesis, we have compared the cellular impact of three highly similar poly(alkyl cyanoacrylate) (PACA) NPs differing only in their alkyl side chains; PBCA (butyl), PEBCA (ethylbutyl) and POCA (octyl), to increase our understanding of the dynamic and varied interactions that arise between NPs and the biological environment.

The first paper investigates the differential activation of the *integrated stress response* (ISR), *unfolded protein response* (UPR) and *antioxidant response* with relation to NP-induced cytotoxicity and mode of cell death. All the PACA variants activated the ISR with the common effect of eIF2α phosphorylation attenuating global protein translation, and accumulation of the transcription factor ATF4. The POCA NPs and high concentrations of the PEBCA NPs induced ER stress seen by activation of the UPR. Both the PEBCA and PBCA NPs was found to induce oxidative stress as observed by increased intracellular ROS, wherein the PBCA NPs was further found to deplete the cellular GSH reserves. The differential induction of cellular stress translated into corresponding differences in cytotoxicity and mode of cell death. POCA generally induced the highest cytotoxicity, and the severity of the insult induced apoptosis without engaging pro-survival responses. PEBCA induced very low cytotoxicity, and this was attributed to a potent pro-survival accumulation of ATF4 and more importantly Nrf2, upregulating key players in the antioxidant response. Upregulation of the cystine transporter SLC7A11, was found to be of high significance. Although PBCA also induced pro-survival ATF4 and Nrf2 signaling it was unable to inhibit ROS propagation, depleting the cells of GSH

and inducing cytotoxicity downstream of the oxidative stress. Interestingly, both PEBCA and PBCA was found to induce the cell death mechanism *ferroptosis* in a manner dependent on the cell's ability to mount a redox defense. Consequently, the PEBCA- and PBCA-induced cytotoxicity was demonstrated to be highly dependent on the level of cystine and transcription of the cystine transporter *SLC7A11*.

The second paper investigate how the PACA variants affect *autophagy*, a catabolic process degrading cytoplasmic material important for maintaining cellular homeostasis and determining cell fate in response to stress. We found that treatment with the PEBCA NPs elevated LC3-II protein levels and upregulate *LC3B* mRNA expression via ISR-activation and ATF4, in a manner that did not alter LC3 flux or autophagic cargo degradation. PBCA also upregulated *LC3B* mRNA levels via the ISR and ATF4, but surprisingly did not elevate LC3-II protein levels. Conversely, PBCA led to an inhibition in LC3 lipidation and autophagic cargo degradation. Treatment with the POCA NPs strongly accumulated LC3-II protein levels without an effect on *LC3B* mRNA levels. The accumulation was found to be associated with reduced degradation of LC3-II as autophagic cargo degradation was inhibited by POCA.

The third paper investigate how the PBCA NPs, specifically, regulates the autophagy pathway by establishing and comparing several functional autophagy assays measuring turnover of bulk autophagic cargo. LC3 as an autophagosome marker is often used to make conclusions on how NPs regulate autophagy, without investigating functional turnover resulting in poorly understood mechanisms. We here demonstrate that the PBCA-induced redox imbalance stimulates autophagic cargo degradation at moderate levels and inhibit autophagic cargo degradation at high levels. The autophagy stimulation was found to depend on JNK1 and p38 MAPK activation, most likely activating autophagy through phosphorylation of Beclin 1 Ser90. Furthermore, we show that PBCA caused a redox imbalance-dependent inhibition in LC3 lipidation that is regulated independently of the regulation of autophagic cargo degradation. At the turning point concentration of PBCA, LC3 lipidation is reduced, but cytoprotective autophagy is still increased in the fight to regain homeostasis.

This work emphasize how the specific NP composition can dictate the outcome of the NP-cell interaction having consequences for cytotoxicity and mode of cell death. By elucidating the molecular mechanisms whereby NPs induce stress, one can better exploit those mechanisms to predict possible *in vivo* toxicities and fine-tune the treatment effect.

SUMMARY IN NORWEGIAN

Nanopartikler benyttes innen utvikling av «drug delivery»-systemer for å øke biotilgjengelighet og minske uønskede bivirkninger hos pasienten. Nanopartikler er av størrelse plassert mellom «bulk»- og molekylære strukturer. Dette gir dem unike egenskaper sammenlignet med deres bulk-motparter. Ved interaksjon med levende celler kan nanopartiklers endrede egenskaper føre til uønskede og uforutsette fysiologiske følger.

For å minske avstanden mellom utvikling av nye nanopartikkel-baserte medisiner og klinisk bruk, er det nødvendig å forstå konsekvensene av deres interaksjon med biologiske systemer. Nanopartiklers fysiske og kjemiske egenskaper som størrelse, form og overflatelading, er avgjørende for hvor kompatible de er med biologiske systemer. I biologiske væsker adsorberer proteiner til nanopartikkel-overflaten og danner en proteinkorona rundt nanopartikkelen. Dette definerer den biologiske identiteten til nanopartikkelen, og utgjør det som registreres av det funksjonelle maskineriet til celler. For å opprettholde normal funksjon og integritet må celler tilpasse seg og respondere på endringer i omgivelsene, som for eksempel behandling med nanopartikler. I denne oppgaven har vi undersøkt cellulær påvirkning ved behandling med tre veldig like poly(alkylcyanoakrylat) (PACA) nanopartikler, som kun skiller seg fra hverandre ved at de har forskjellige alkylsidekjeder; PBCA (butyl), PEBCA (etylbutyl) og POCA (oktyl). Dette for å øke vår forståelse av de dynamiske og varierte interaksjonene som oppstår mellom nanopartikler og det biologiske miljøet.

Den første artikkelen undersøker aktivering av *integrert stressrespons* (ISR), *ufoldet proteinrespons* (UPR) og *antioksidantrespons* i forhold til nanopartikkel-indusert cytotoksisitet og type celledød. Alle PACA-variantene aktiverte ISR som fører til fosforylering av eIF2α, attenuering av global proteintranslasjon og akkumulering av transkripsjonsfaktoren ATF4. POCA og høye konsentrasjoner av PEBCA induserte ER-stress, observert ved aktivering av UPR. Både PEBCA og PBCA induserte oksidativt stress, observert ved økt intracellulær ROS, hvorpå PBCA i tillegg tømte de cellulære GSH-reservene. Den differensielle induksjonen av cellulært stress gir tilsvarende forskjeller i cytotoksisitet og type celledød. POCA induserte generelt den høyeste cytotoksisiteten og apoptose uten å indusere «pro-survival» responser. PEBCA induserte svært lav cytotoksisitet, og dette ble tilskrevet en kraftig «pro-survival» akkumulering av ATF4 og enda viktigere Nrf2, som oppregulerte nøkkelfaktorer innen antioksidantresponsen. Oppregulering av cystintransportøren SLC7A11 ble funnet å være av stor betydning. Selv om PBCA også induserte pro-survival ATF4- og Nrf2-signalering, var

cellene ikke i stand til å hemme ROS-dannelse eller tap av GSH, og PBCA førte til cytotoksisitet nedstrøms for oksidativt stress. Et svært interessant funn var at både PEBCA og PBCA induserte celledødsmekanismen *ferroptose*, avhengig av cellens evne til å understøtte et redoksforsvar. Følgelig ble det vist at PEBCA- og PBCA-indusert cytotoksisitet var sterkt avhengig av nivået av cystin og transkripsjon av cystintransportøren SLC7A11.

Den andre artikkelen undersøker hvordan PACA-variantene påvirker *autofagi*, en prosess som bryter ned cytoplasmatisk materiale og er viktig for å opprettholde cellulær homeostase. Vi fant at behandling med PEBCA økte LC3-II proteinnivået og oppregulerte *LC3B* mRNA-ekspresjon via ISR-aktivering og ATF4 på en måte som ikke endret LC3-fluks eller degradering ved autofagi. PBCA oppregulerte også *LC3B* mRNA-nivåer via ISR og ATF4, men økte overraskende nok ikke LC3-II-proteinnivåer. I motsetning førte PBCA til en hemming av LC3-lipidering og degradering ved autofagi. Behandling med POCA medførte kraftig akkumulering av LC3-II proteinnivåer uten å påvirke *LC3B* mRNA-nivåer. POCA ble funnet å hemme degradering ved autofagi og av den grunn akkumulere LC3-II.

Den tredje artikkelen undersøker hvordan PBCA spesifikt regulerer autofagi ved å etablere og sammenligne flere funksjonelle autofagi-analyser som måler omsetningen av autofagisk «bulk cargo». LC3 som autofagosom-markør brukes ofte til å avgjøre hvordan nanopartikler regulerer autofagi, uten å undersøke funksjonell degradering, noe som igjen resulterer i en dårlig forståelse av mekanismene. Vi viser i dette arbeidet at PBCA-indusert redoks-ubalanse stimulerer degradering ved autofagi ved moderate nivåer og hemmer degradering ved autofagi ved høye nivåer. Stimuleringen av autofagi ble funnet å være avhengig av JNK1 og p38 MAPK-aktivering, mest sannsynlig gjennom fosforylering av Beclin 1 Ser90. Videre viser vi at PBCA forårsaket en redoks ubalanse-avhengig hemming i LC3-lipidering som er regulert uavhengig av degraderings reguleringen. Ved vendepunkts-konsentrasjonen av PBCA, reduseres LC3-lipidering, men cytoprotektiv autofagi økes fortsatt i kampen for å gjenvinne homeostase.

Dette arbeidet viser tydelig hvordan den spesifikke nanopartikkel-sammensetningen kan være bestemmende for utfallet av nanopartikkel-celleinteraksjonen, som igjen får konsekvenser for cytotoksisitet og type celledød. Ved å belyse de molekylære mekanismene hvormed nanopartikler induserer stress, kan man bedre utnytte disse mekanismene til å predikere mulig *in vivo*-toksisitet og finjustere effekten av behandlingen.

ABBREVIATIONS

AMP	5′ adenosine monophosphate
AMPK	(5′ adenosine monophosphate)-activated protein kinase
AP-1	Activator protein-1
ARE	Antioxidant response element
ATF/CREB	Activating transcription factor/cyclic AMP response element binding protein
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor-6
ATG	Autophagy-related
BafA1	Bafilomycin A1
BH3	Bcl-2 homology domain
bZIP	Basic leucine zipper domain
CaMKII	Calcium/calmodulin-dependent protein kinase II
CHOP	C/EBP-homologous protein
CMA	Chaperone-mediated autophagy
CMV	Cytomegalovirus
CReP	Constitutive repressor of eIF2α phosphorylation
DAPK1	Death-associated protein kinase-1
DFCP1	Zinc finger FYVE domain-containing protein 1
EBSS	Earle's balanced salt solution
eIF2α	α subunit of eukaryotic translation initiation factor-2
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
ESCRT-III	Endosomal sorting complex required for transport III
GABARAP	Gamma-aminobutyric acid receptor-associated protein
GADD34	Growth arrest and DNA damage-inducible protein
GCN2	General control non-derepressible-2
GPX4	Glutathione peroxidase 4
GRP78	78-kDa glucose-regulated protein
GSH	Glutathione
GSSG	Oxidized GSH
HRI	Heme-regulated inhibitor

IRE1	Inositol-requiring enzyme-1
ISR	Integrated stress response
JNK	c-jun NH_2-terminal kinase
Keap1	Kelch-like ECH-associated protein 1
LDH	Lactate dehydrogenase
LDHB	Lactate dehydrogenase B
LIR	LC3-interacting region
LLPD	Long-lived protein degradation assay
MAPKs	Mitogen-activated protein kinases
MDA	Malondialdehyde
mKeima	Monomeric Keima
Mst1	Mammalian STE20-like protein kinase 1
mTOR	Target of rapamycin
mTORC1	Target of rapamycin complex 1
NF-kB	Nuclear factor kappa B
NPs	Nanoparticles
Nrf2	Nuclear factor erythroid 2 (NF-E2)-related factor 2
p-eIF2α	Phosphorylated α subunit of eIF2
PACA	Poly(alkyl cyanoacrylate)
PBCA	Poly(butyl cyanoacrylate)
PE	Phosphatidylethanolamine
PEBCA	Poly(ethylbutyl cyanoacrylate)
PEG	Polyethylene glycol
PERK	Protein kinase RNA (PKR)-like ER kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PI3K	Phosphatidylinositol 3-kinase Class I
PI3KC3	Phosphatidylinositol 3-kinase Class III
PKR	Protein kinase double-stranded RNA-dependent
POCA	Poly(octyl cyanoacrylate)
PP1	Protein phosphatase 1 complex
RIDD	Regulated IRE1-dependent decay
ROS	Reactive oxygen species
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

siRNAs	Small interfering RNAs
TEM	Transmission electron microscopy
TFEB	Transcription factor EB
tRNAs	transfer RNAs
ULK1	Unc-51-like kinase 1
UPR	Unfolded protein response
WIPI	WD-repeat protein-interacting phosphoinositide
XBP1	X-box-binding protein 1
αMSH-PEG-C′ dots	PEG-coated silica NPs functionalized with a melanoma-targeting peptide

LIST OF PUBLICATIONS INCLUDED IN THIS THESIS

I. **Small variations in nanoparticle structure dictate differential cellular stress responses and mode of cell death.**
Szwed M*, Sønstevold T*, Øverbye A, Engedal N, Grallert B, Mørch Ý, Sulheim E, Iversen TG, Skotland T, Sandvig K, Torgersen ML.
Nanotoxicology. 2019 Aug;13(6):761-782. * shared first author

II. **Structural Variants of poly(alkylcyanoacrylate) Nanoparticles Differentially Affect LC3 and Autophagic Cargo Degradation.**
Sønstevold T, Engedal N, Mørch Ý, Iversen TG, Skotland T, Sandvig K, Torgersen ML.
J Biomed Nanotechnol. 2020 Apr 1;16(4):432-445.

III. **Perturbation of cellular redox homeostasis dictates divergent effects of polybutyl cyanoacrylate (PBCA) nanoparticles on autophagy**
Sønstevold T, Engedal N, Torgersen ML.
Manuscript

Related publications not included in this thesis

I. **Ceramide-containing liposomes with doxorubicin: time and cell-dependent effect of C6 and C12 ceramide.**
Øverbye A, Holsæter AM, Markus F, Škalko-Basnet N, Iversen TG, Torgersen ML, Sønstevold T, Engebraaten O, Flatmark K, Mælandsmo GM, Skotland T, Sandvig K.
Oncotarget. 2017 Aug 12;8(44):76921-76934.

II. **Cabazitaxel-loaded poly(alkyl cyanoacrylate) nanoparticles: Toxicity and changes in the proteome of breast, colon and prostate cancer cells**
Øverbye A, Torgersen ML, Sønstevold T, Iversen TG, Mørch Ý, Skotland T, Sandvig K. *Nanotoxicology. Accepted 2021 Apr. DOI: 10.1080/17435390.2021.1924888*

INTRODUCTION

The general idea that a drug's performance could be improved if the active molecule can reach and interact better with its target site was established at the beginning of the twentieth century by Paul Ehrlich's concept of the 'Magic bullet'. This idea emerged from the understanding of the antibody-antigen specificity related to infectious diseases (Strebhardt et al. 2008). Since then, delivering drugs to their site of action to achieve a better therapeutic outcome has evolved into a vast research field and 'big pharma'. Drug delivery systems are developed to maximize drug efficacy and minimize side effects for the patient. Nanotechnology holds significant promise to improve treatment and prevention of disease, by providing vehicles for targeted therapy. To understand the true potential and limitations of nanoparticles (NPs) as drug delivery systems, we need to decipher the consequences of their interactions with biological systems. This work addresses the variation in cellular stress responses that can arise from even subtle differences in the polymer composition of poly(alkyl cyanoacrylate) (PACA) NPs.

NANOPARTICLES IN DRUG DELIVERY

The prefix 'nano' has become a popular label in modern science, arising from the characteristics of nanoscale structures. The nanometer is a metric unit length equal to one-billionth of a meter (10^{-9} m). Nanotechnology includes all types of research and technologies that deal with the unique properties of matter that occur below a given size threshold. In effect, nanomaterials are according to their size positioned between bulk structures and atomic or molecular structures, enabling unique applications of the materials (Buzea et al. 2007). One characteristic feature is the increase in surface area to volume ratio, altering the material's mechanical, thermal, and catalytic properties compared to known bulk properties. Nanomedicine exploits these unique physico-chemical characteristics in tissue engineering, or to develop biosensors and drug delivery systems (Pelaz et al. 2017).

The general purpose of a drug carrier is to provide the drug with a more appropriate or attractive pharmacokinetic and biodistribution profile, promoting biological efficacy and reducing side effects by giving a more efficient distribution at the target site (Vauthier 2019). This may be achieved by exploiting the unique properties of NPs. NP encapsulation may increase the

solubility and stability of a drug, enabling increased circulation times and bioavailability. They can be designed to improve specificity of distribution and bypass important physiological barriers. All of which will promote a greater accumulation at the target site, making it possible to reduce the administrated dose and in turn limit side effects (Blanco et al. 2015).

In the infancy of the research field in the early 1970s, small vesicles called liposomes were used to encapsulate and improve the performance of existing drugs. As opposed to covalently linking the active ingredient to its carrier, entrapment within a vesicle kept the chemical structure unaltered and allowed for more molecules to be packaged within each vesicle (Vauthier 2019). Today, NPs have revolutionized drug delivery and they are extensively studied as carriers of genes or drugs, as sole therapeutic agents, and even as multifunctional particles combining a diagnostic and therapeutic function (Pelaz et al. 2017). Perhaps the prime example of NPs as delivery vehicles today is the Covid-19 vaccine. Several pharmaceutical companies have successfully based their vaccine on intracellular delivery of the SARS-CoV-2 spike protein mRNA by lipid NPs (Polack et al. 2020, Baden et al. 2021).

Polymer nanoparticles

Compared to the early liposomes, polymer NPs were more stable in biological media, provided a higher loading capacity and greater possibility for sustained drug-release (Zheng et al. 2016). However, to synthesize polymer NPs compatible with clinical application, NPs were made from the polymerization of alkyl cyanoacrylate monomers already used as surgical glue. This provided a biodegradable polymer NP that was further demonstrated to be able to associate drugs and function as lysosomotropic carriers (Couvreur et al. 1979). Today, biodegradable PACA NPs having distinct features can be obtained by various polymerization and spontaneous emulsification techniques using a variety of monomers to titrate the wanted properties (Landfester 2006, Nicolas et al. 2009). A general PACA NP is illustrated in Figure 1 and Figure 10. The biodegradable PACA polymers are hydrophobic, and as drugs are not covalently linked but associated through hydrophobic interaction the PACA NPs are especially suitable for encapsulating hydrophobic drugs (Nicolas et al. 2009).

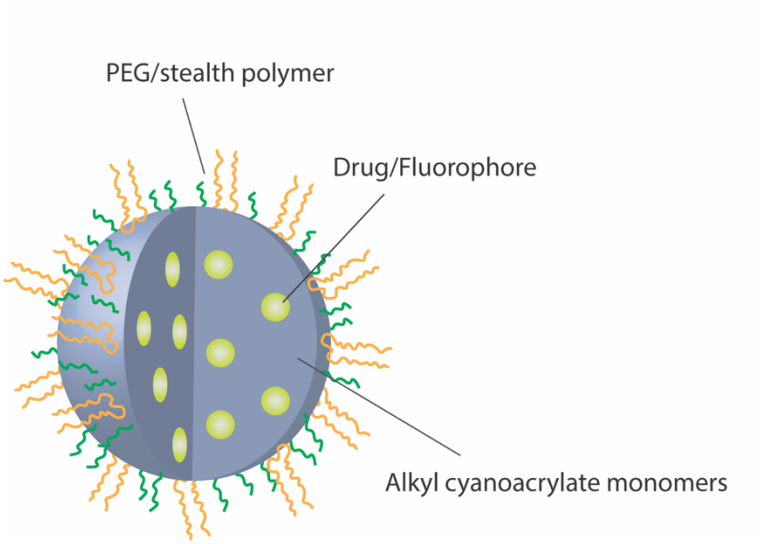

Figure 1. Schematic presentation of a PACA NP coated with a stealth polymer.

Efficient drug delivery requires both NP uptake by cells and release of the drug once inside the cell. Clathrin-mediated endocytosis is the predominant cellular uptake mechanism of PACA NPs, although caveolin-mediated endocytosis is also found to be involved (Iversen et al. 2011, Sulheim et al. 2016). Inside the late endosomes, hydrolysis of the ester bond of the alkylcyanoacrylate (the alkyl side chain of the polymer) is described as the primary mechanism causing dissolution of the PACA NPs and release of drugs concomitant to NP degradation *in vivo*. Resultantly, degradation of PACA NPs leads to the water-soluble alkyl alcohol and poly(cyanoacrylic acid) that can be renally excreted (Vauthier et al. 2003, Graf et al. 2009). An important proof of concept that degradable PACA NPs could reduce the toxicity of a drug while providing a therapeutic effect was illustrated with the anticancer drug doxorubicin. The cardiotoxicity of doxorubicin, initially limiting patient treatment, was decreased by entrapment within PACA NPs due to low uptake in myocardium. In addition, a significant reduction in both mortality and weight loss of the mice was obtained (Couvreur et al. 1982). Furthermore, poly(2-ethyl-butyl cyanoacrylate) NPs loaded with cabazitaxel showed longer circulation time in blood, higher drug concentration in tumor and higher infiltration of anti-tumorigenic macrophages compared to free cabazitaxel, increasing treatment efficacy in basal-like patient-derived xenografts (Fusser et al. 2019).

Mechanisms of nanoparticle-induced toxicity

While considerable progress has been made in understanding NPs as drug carriers, many challenges remain to accelerate translation into clinical use. Upon interaction with living cells the altered properties of nanomaterials, such as surface reactivity, may lead to undesired and unforeseen physiological consequences. The NP physicochemical characteristics, such as size, shape, surface charge, and coating, play key roles in determining the biocompatibility of NPs (Savage et al. 2019). Adsorption of proteins onto surfaces was demonstrated already in 1962 by Leo Vroman (Vroman 1962), and the continuous flux of adsorption and desorption of proteins (Vroman et al. 1980) was later termed the 'Vroman effect'. As depicted in Figure 2, a phenomenon referred to as a *protein corona* forms around NPs in biological fluids as proteins compete for the NP surface. The rate, affinity and stoichiometry of protein association and dissociation defines the NP surface in the end; the biological identity of the NP (Cedervall et al. 2007). The association and dissociation kinetics between the proteins and the NP surface divide the protein corona into two layers, the 'hard' and the 'soft' corona. Proteins which bind with high affinity and display high association rates interact tightly with the NP surface and constitute the hard corona, whereas proteins with low binding affinity and low association rates interact more loosely with the NP surface and constitute the soft corona (Cedervall et al. 2007, Monopoli et al. 2012, Wolfram et al. 2014, Ahsan et al. 2018). The selective adsorption of proteins on the NP surface has been shown to be determined by a complex interplay between the NP's synthetic characteristics and the relative concentrations of different proteins in the biological fluid (Maiorano et al. 2010, Tenzer et al. 2011, Zhang et al. 2011, Ghavami et al. 2013, Mohammad-Beigi et al. 2020). The protein corona consists in large parts of opsonins, marking the NPs for rapid clearance by phagocytosis (Cai et al. 2020). This largely affect NP circulation time, and a common approach to overcome this is to coat the synthetic surface of the NP with a 'stealth' polymer, such as polyethylene glycol (PEG), reducing interactions with the components of biological fluids. Such PEGylation is demonstrated in Figure 1. Moreover, the PEG coat also shields NPs from surface aggregation as uncoated NPs often have hydrophobic surfaces (Suk et al. 2016). Effectively, for coated NPs, the coat is an essential determinant for the exact interaction with the various components of biological fluids.

Importantly, the specifically acquired protein corona defines the NP's biological identity, and hence, what is seen by the functional machinery of cells, affecting cell internalization, biodistribution and cytotoxicity (Owens et al. 2006, Lundqvist et al. 2008,

Aggarwal et al. 2009). The density and thickness of the protein corona determines overall size of the NP and level of exposure of the underlying synthetic NP surface. The identity and orientation of adsorbed proteins influence the array of possible biological interactions and the accessibility of potential binding- and catalytic-domains. Furthermore, the protein conformation affects protein activity and its interaction with other biomolecules (Walkey et al. 2012, Liu et al. 2020). As illustrated in Figure 2, the interface between NPs and biological systems consists of the NP surface (the NP's synthetic characteristics, Figure 2A), the solid-liquid interface (the NP's biological characteristics defined by the protein corona, Figure 2C), and the contact zone with the biological substrate (the interaction between the NP's biological identity and cells, Figure 2B) (Nel et al. 2009). Cell membranes are self-assembled lipid bilayers, wherein the shape of the lipid species and membrane curvature govern transmembrane structures, membrane permeability and enzyme activation (Mouritsen 2011). Proteins, cholesterol and lipopolysaccharides in the lipid bilayer allow the cell to respond to the external environment and communicate with other cells (Gatenby 2019). The non-covalent electrostatic forces, hydrophobic interactions and hydrogen bonding are the main forces driving NP-cell contact, further ligand-receptor interactions increase the complexity of the contact site (Nel et al. 2009, Wang et al. 2019). The nature of the NP surface facilitates the interaction with charged regions or binding to surface ligands of the cell membrane, and may induce conformational changes due to changes in free surface energy or oxidant injury due to increased ROS (Nel et al. 2006). The NP-cell interaction may lead to alterations in membrane fluidity, the composition of micro-domains, or membrane curvature (Nel et al. 2009). Such membrane alterations are known to affect the activity of membrane proteins like receptors, enzymes, ion channels, and nutrient transporters, and have been proposed to signal membrane stress to the cell interior (Mouritsen 2011). The nature and extent of these interactions influence processes such as NP wrapping at the cell surface, endocytosis and intracellular biocatalysis properties, and participate in determining the biocompatibility of a given NP (Nel et al. 2009, Wang et al. 2019).

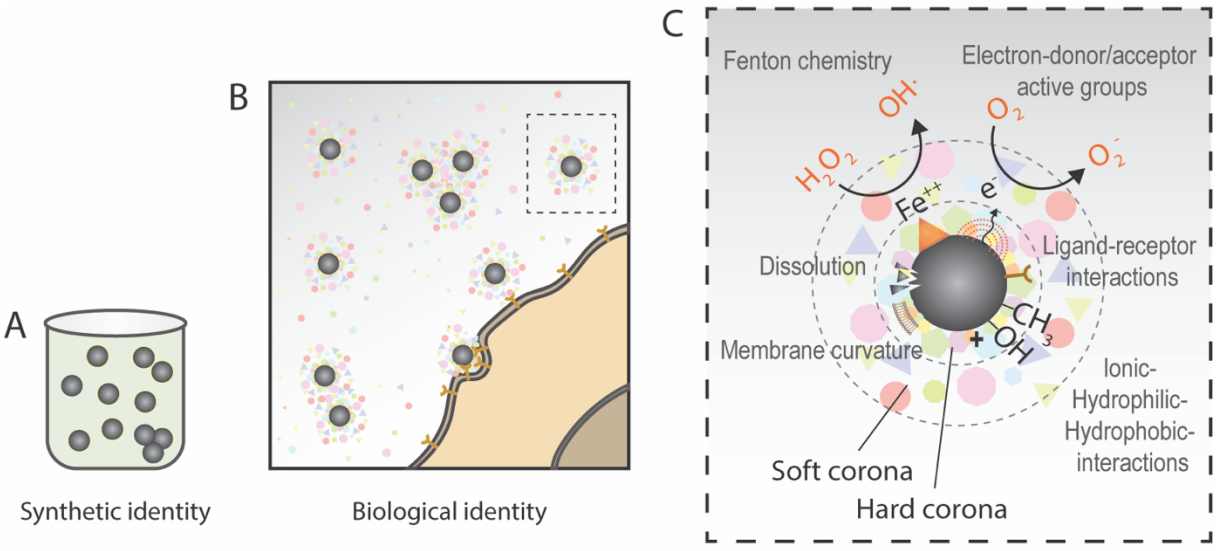

Figure 2. The physicochemical characteristics of the initial NP material make up the synthetic identity of the NP (**A**). These characteristics govern the formation of the protein corona in a biological environment (**B**). Protein attachment/detachment kinetics divide the protein corona into two layers. The hard corona constitutes proteins with high binding affinity, whereas the soft corona constitutes proteins of low binding affinity. The nature of the NP surface drive cell contacts through non-covalent electrostatic, hydrophobic, or hydrophilic interactions, and ligand-receptor binding. Depending on the NP chemical composition, catalytically active or passive surface groups may facilitate electron transfer, metal ions may participate in Fenton reactions, protein interactions may cause dissolution of the NP or NP-cell interactions may lead to membrane alterations (**C**).

A fundamental property of nanomaterials is their ability to facilitate electron transfer, thus promoting oxidative damage or conversely providing antioxidant protection (Nel et al. 2006, Wen et al. 2020). Reactive oxygen species (ROS) are regarded as unavoidable byproducts of aerobic metabolism and are thus continuously generated, transformed, and consumed in all living organisms (Ray et al. 2012). As such, ROS serves as important physiological regulators activating signaling pathways (Finkel 2011, Murphy et al. 2011, Sena et al. 2012). However, when there is an imbalance between ROS production and ROS scavenging, high ROS levels promote oxidative stress potentially damaging proteins, DNA and lipids (Ray et al. 2012, Schieber et al. 2014), as illustrated in Figure 3.

Generation of free radicals and other reactive species, disturbing the existing oxidative balance, are found to be the main cause of NP-induced cytotoxicity and genotoxicity of various NPs (Nel et al. 2006, Fu et al. 2014, Wen et al. 2020). Electron transfer at the nano-bio interface is a complicated process as the redox potentials and energy states of the surface atoms differ with the varying structural features of NPs, such as size, shape, coating, and adsorbed proteins. Additionally, external factors such as the pH of the solution and possible external irradiation

largely impacts the pro- or antioxidant abilities of NPs (Hartmann et al. 2013, Wang et al. 2015, Tian et al. 2018, Wang et al. 2019). As the size of the particle decreases a greater proportion of its atoms or molecules are displayed on the surface rather than interior of the material. The smaller size may increase the number of structural defects which give rise to altered electronic properties and can establish specific surface groups that function as reactive sites. Depending on the particular NP chemical composition this may give hydrophilic or hydrophobic, lipophilic or lipophobic, or catalytically active or passive surface groups (Oberdörster et al. 2005, Nel et al. 2006).

Electron-transfer will occur only if the relevant orbital energies of the electron donors and acceptors are at approximately the same level (Xu et al. 2000). When the energy levels of NPs are lower than the redox potential of reactive species in biological fluids, electrons can directly transfer to the NP and the NP functions as a ROS scavenger mitigating the oxidative stress (Wang et al. 2019). This has been demonstrated for carbon-based NPs such as fullerene (Lao et al. 2009), ceria NPs (Li et al. 2015), and palladium nanocrystals (Ge et al. 2016). Conversely, some NPs have been found to cause reactive species via the surface sorbate obtained through interaction with biological components. Attached molecules can change the surface energy properties of the NP and enable surface atom dissolution or electron donation reducing H_2O, O_2 and H_2O_2 to form $O_2^{\bullet-}$ or $\bullet OH$ (Nel et al. 2006, Kermanizadeh et al. 2015, Wang et al. 2019). This has been well demonstrated for palladium-based nanostructures (Fang et al. 2018). Furthermore, metal ions released by metal oxide NPs are found to promote ROS through participation in redox cycling or catalysis via Fenton reaction [(i) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$, (ii) $Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + \bullet OOH + H^+$] or Fenton-like reactions such as demonstrated for AgNPs [$Ag + H_2O_2 + H^+ \rightarrow Ag^+ + \bullet OH + H_2O$] (Nel et al. 2006, He et al. 2012, Tian et al. 2018). NPs such as silver and silicon dioxide may cause enzyme deactivation or membrane structure disruption which further facilitate ROS generation by impairing mitochondrial respiration, affecting NADPH oxidase or cellular calcium homeostasis (Carlson et al. 2008, Guo et al. 2016, Cui et al. 2020, Lee et al. 2020).

Taken together, the NPs synthetic and biological identity may induce toxicity by a variety of known and unknown mechanisms initiating cellular stress. The cellular stress responses investigated in this thesis are presented in the section below.

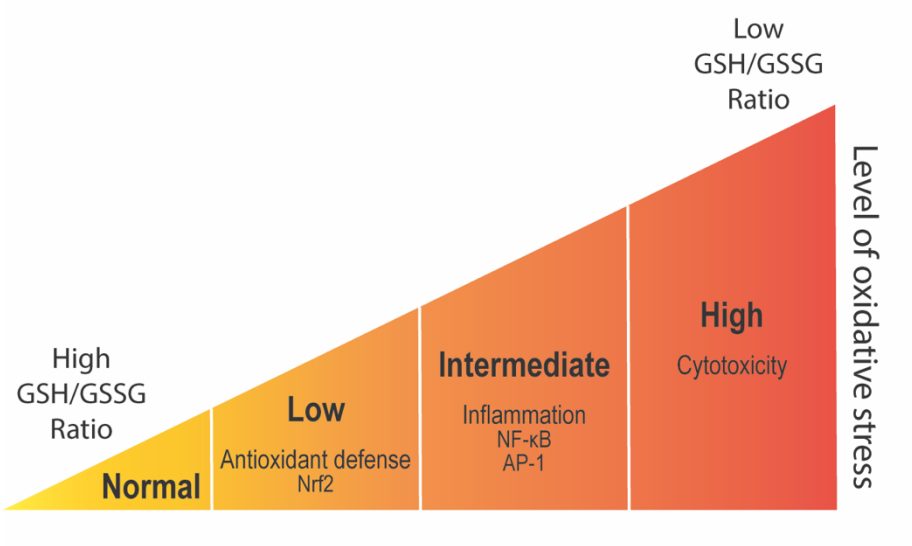

Figure 3. A stratified oxidative stress model. At normal levels, ROS are continuously generated, transformed, and consumed. At low levels, antioxidants are induced via transcriptional activation of the antioxidant response to restore homeostasis. As ROS levels increase this protective response is overtaken by inflammation and finally cytotoxicity damaging proteins, DNA, and lipids.

CELLULAR STRESS RESPONSES

Responding to its continuously changing environment is one of the biggest challenges for cells. To maintain proper function and integrity, the cell needs to adapt to fluctuations in external conditions (such as temperature and ultraviolet light), in chemical signals (such as ion concentrations, redox potentials and levels of metabolic intermediates or byproducts), in extracellular signals (such as hormones and cytokines), and pathogen invasions. Beyond a certain threshold, these fluctuations are perceived as stress, and the cell's ability to respond determines its fate. Consequently, an intricate surveillance network is put in place for the cell to protect itself from unfavorable conditions (Chen et al. 2010, Kroemer et al. 2010).

The stress induced by NPs has the potential to initiate a whole spectrum of cellular stress responses with the aim to regain homeostasis. Changes in a range of physiological and pathological conditions activate the *integrated stress response* (ISR), while endoplasmic reticulum (ER) stress activates the *unfolded protein response* (UPR) more specifically. An increase in ROS or reduction of ROS scavengers may activate the *antioxidant response* mediated by transcription factors such as Nrf2 (nuclear factor erythroid 2 (NF-E2)-related factor 2) and ATF4 (activating transcription factor 4), while at the same time engaging the ISR and

autophagy. Autophagy is induced to clear dysfunctional components or recycle unnecessary components when nutrient availability is scarce. While these cellular stress responses initially work as pro-survival pathways, they may also facilitate cellular death if the insult is prolonged or too severe.

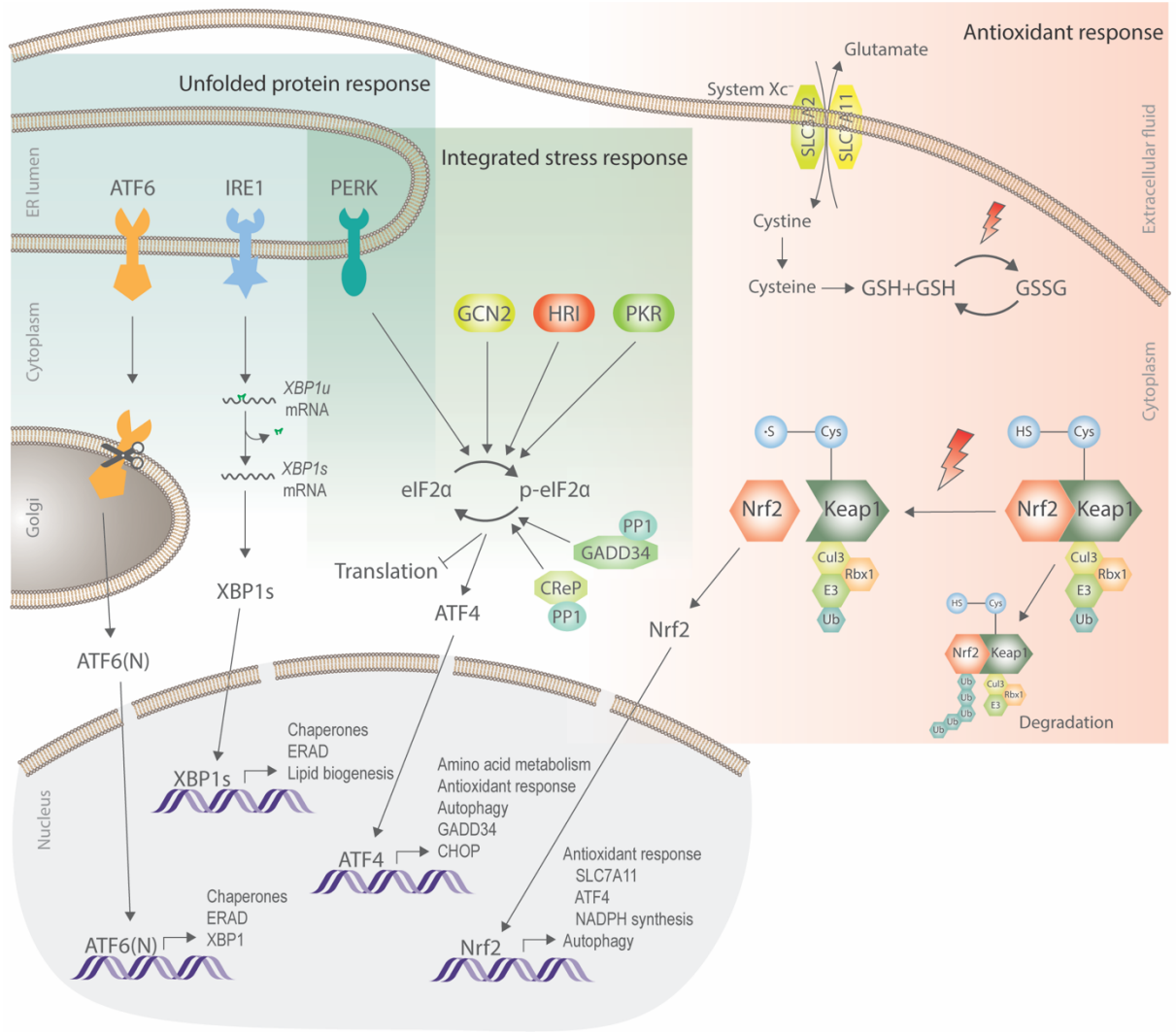

Figure 4. In mammals the UPR includes three separate signal transduction pathways downstream of three ER transmembrane protein sensors, namely ATF6, IRE1, and PERK. The luminal domains of these ER stress transducers sense the protein folding status and transmit the information to the cytoplasmic domains, interacting with the transcriptional and translational apparatus. The PERK pathway of the UPR is also known as part of the ISR. Changes in a range of physiological and pathological conditions activate the ISR. The common point of convergence is phosphorylation of eIF2α, downregulating global protein synthesis while at the same time allowing translation of selected genes important for cell survival. In the antioxidant response, GSH is considered the most abundant antioxidant enabling electron donation as two electron-donating GSH molecules form oxidized GSSG. Cystine import through the cystine/glutamate antiporter system X_C^- is a crucial factor regulating intracellular levels of cysteine, a precursor for GSH synthesis, hence maintaining cellular GSH levels

and redox balance. A major regulator of cytoprotective responses to oxidative stress is the Nrf2-Keap1 pathway. Keap1 targets Nrf2 for ubiquitination and subsequent degradation by a Cullin-3-mediated ubiquitination complex, keeping Nrf2 at low levels under normal conditions. When cells are exposed to oxidative stress, oxidation of reactive cysteine residues in Keap1 inactivates Keap1 resulting in Nrf2 stabilization, translocation to the nucleus and upregulation of cytoprotective genes.

The unfolded protein response

The ER does not only serve as a 'protein folding factory' coordinating folding and post-translational modifications, but participates in various fundamental functions including calcium homeostasis, glucose concentration regulation, and lipid metabolism (Liu et al. 2015, Hetz et al. 2020). Consequently, a fully functional ER is vital for the cell.

During translation, the linear sequence of amino acids rapidly starts the protein folding process to fulfill thermodynamic and kinetic requirements, burying nonpolar residues in the core of the molecule. Rate-limiting reactions in the folding process are further accelerated by folding enzymes, such as protein disulfide isomerases, while proteins acting as molecular chaperones, such as small heat shock proteins, prevent aggregation and facilitate protein maturation (Hebert et al. 2007). As virtually all signaling proteins that the cell uses to communicate with its environment are assembled in the ER, only properly folded proteins should be able to advance from the ER. When ER is challenged by physiological demands or pathological insults, such as perturbations in cellular energy, calcium homeostasis or redox status, accumulation of unfolded or misfolded proteins causes a condition referred to as 'ER stress' (Hebert et al. 2007, Hetz et al. 2020). Accumulation of misfolded proteins is harmful to the cell, and thus mechanisms designed to detect such proteins and either refold them or target them for degradation have evolved. To ensure protein folding fidelity and maintain ER functions, ER stress engages the UPR (Hebert et al. 2007, Hetz et al. 2020). The UPR aims to restore normal function by halting protein translation lessening the protein processing load for the ER, and upregulating genes involved in protein folding such as ER chaperones and folding enzymes, as well as components of the ER-associated degradation (ERAD) pathway (Hetz et al. 2020). Protein folding and degradation is strongly interconnected, and proteins that cannot be refolded to their correct conformation is ubiquitinated and targeted for proteasomal degradation by the ERAD pathway (Hebert et al. 2007). Resultantly, increased protein ubiquitination and expression of heat shock proteins are markers of ER stress. However, if the cell is not able to regain ER function or the

disruption is prolonged, the UPR initiate apoptosis in a model proposed to depend on death threshold levels of CHOP (C/EBP-homologous protein) and GADD34 (growth arrest and DNA damage-inducible protein) (Ron et al. 2007).

In mammals the UPR includes three separate signal transduction pathways downstream of three ER transmembrane protein sensors, namely the inositol-requiring enzyme-1 (IRE1), the activating transcription factor-6 (ATF6), and the protein kinase RNA (PKR)-like ER kinase (PERK). The luminal domains of these ER stress transducers sense the protein folding status and transmit the information to the cytoplasmic domains, interacting with the transcriptional and translational apparatus (Ron et al. 2007), as illustrated in Figure 4.

IRE1 oligomerizes in the ER membrane upon direct binding of unfolded proteins to its luminal domain. This allows for *trans*-autophosphorylation of the IRE1 kinase domain, which acts as a site-specific endonuclease to cleave the mRNA encoding the transcription factor X-box-binding protein 1 (XBP1) (Calfon et al. 2002). This results in the expression of an active XBP1 transcription factor that upregulates genes involved in ER protein translocation, folding and secretion, and degradation of misfolded proteins (Hetz et al. 2020). IRE1 can also cleave a small set of mRNAs or precursor microRNAs in a process known as regulated IRE1-dependent decay (RIDD), thought to relieve protein folding load by lowering mRNA abundance (Hollien et al. 2006).

ATF6 is synthesized as an inactive precursor, tethered to the ER membrane by its transmembrane segment. Upon ER stress, ATF6 transits from ER to the Golgi apparatus, where it is cleaved to release the active cytosolic DNA-binding fragment, termed ATF6p50 (Haze et al. 1999). ATF6p50 translocates to the nucleus where it acts both in parallel and overlapping with XBP1, upregulating ER protein folding capacity as well as promoting ER and Golgi apparatus biogenesis to increase secretory capacity (Hetz et al. 2020).

The ER luminal domain of PERK is normally bound by the 78-kDa glucose-regulated protein (GRP78). In the classical model of PERK activation, GRP78 dissociates from PERK upon an accumulation of misfolded proteins, allowing *trans*-autophosphorylation of its kinase domain (Bertolotti et al. 2000), and thereby gaining full catalytic activity to phosphorylate the α subunit of eukaryotic translation initiation factor-2 (eIF2α) on serine 51 (Ernst et al. 1979, Pathak et al. 1988, Harding et al. 1999). However, due to its strong sequence homology with IRE1, PERK has also been proposed to be activated by direct binding of unfolded proteins to its luminal domain (Korennykh et al. 2012). eIF2 participates in translation initiation by forming a 43S preinitiation complex, and when bound to GTP facilitates recognition of the

initiation codon by this complex (Jackson et al. 2010). Phosphorylation of the α subunit of eIF2 (p-eIF2α) inhibits the guanine nucleotide exchange factor eIF2B, which catalyze the exchange of GDP-bound eIF2 to its active form GTP-bound eIF2. Consequently, p-eIF2α reduce the amount of active eIF2 resulting in lower levels of translation initiation and, hence, global reduction in the load of newly synthesized proteins (Kimball 1999, Jackson et al. 2010). In addition to reducing protein load for the ER, PERK-mediated p-eIF2α also contributes to transcriptional upregulation of specific mRNAs that harbor one or more upstream open reading frames in their 5′ untranslated regions, whereof ATF4 is the best characterized (Lu et al. 2004). Additional eIF2 kinases exist that can activate this pathway independent of ER stress. Hence, the PERK pathway of the UPR is also known as part of the ISR (Figure 4).

The integrated stress response

The ISR is an elaborate signaling pathway in eukaryotic cells activated in response to both extrinsic conditions, such as hypoxia and amino acid deprivation, and intrinsic conditions, such as accumulation of misfolded proteins. The common point of convergence is phosphorylation of the serine 51 residue of eIF2α (Ron 2002, Pakos-Zebrucka et al. 2016), downregulating global protein synthesis while at the same time allowing translation of selected genes important for cell survival (Lu et al. 2004).

As illustrated in Figure 4, four serine/threonine kinases catalyze the phosphorylation of eIF2α; PERK (as already addressed under UPR), PKR (protein kinase double-stranded RNA-dependent), GCN2 (general control non-derepressible-2) and HRI (heme-regulated inhibitor). They have a high degree of sequence similarity, underlying their common role in phosphorylating eIF2α. However, each kinase primarily responds to a distinct type of stress, reflecting their unique regulatory mechanisms (Donnelly et al. 2013). While PERK is primarily activated by ER stress, PKR is primarily activated by double-stranded RNA during viral infections, GCN2 in response to amino acid deprivation and HRI by heme deficiency and oxidative stress (Pakos-Zebrucka et al. 2016). Although distinct types of stress activate the eIF2a kinases, they have overlapping functions and may act cooperatively or compensate for each other to precisely tune the cellular response (Ron 2002, Donnelly et al. 2013, Pakos-Zebrucka et al. 2016).

The PKR protein is localized in the cytosol and nucleus, and contains an N-terminal double-stranded RNA binding domain and a C-terminal kinase domain. Dimerization of the kinase domain upon binding of double-stranded RNA leads to autophosphorylation and subsequent functional activation of the kinase (Ung et al. 2001, Vattem et al. 2001, Lemaire et al. 2008). As an important strategy to inhibit viral replication, PKR activation leading to p-eIF2α blocks translation of viral mRNAs. PKR activation has also been implicated as an activator of signaling pathways such as p53 and NF-κB, mediating PKR-induced apoptosis (García et al. 2007).

Activation of GCN2 is proposed to depend on the accumulation of deacylated transfer RNAs (tRNAs) that occur when cells are starved for amino acids as tRNA synthetase enzymes fail to aminoacylate tRNA (Wek et al. 1989, Masson 2019). The binding of deacylated tRNAs to the histidyl-tRNA synthetase-related domain on GCN2 is thought to release the autoinhibitory interactions of the inactive GCN2 homodimer (Dong et al. 2000). This allows for autophosphorylation and kinase activation, attenuating general protein translation when amino acids are scarce. As an additional mechanism, independent of deacylated tRNA, stalled ribosomes are described to mediate GCN2 activation (Ishimura et al. 2016), and GCN2 is further found to be activated by glucose deprivation (Ye et al. 2010), viral infections (Berlanga et al. 2006) and UV irradiation (Grallert et al. 2007).

HRI was initially thought to mainly be expressed in erythroid cells, protecting the cell against toxic accumulation of α- and β-globin when heme levels are low under iron deficiency (Kramer et al. 1976, Levin et al. 1976, Chen 2006). Binding of heme to the two heme-binding domains of HRI causes a stable, inactive HRI dimer when cellular heme levels are high. In the absence of heme, the HRI dimer autophosphorylates activating its kinase domain attenuating general protein translation including globin mRNA (Rafie-Kolpin et al. 2000, Bauer et al. 2001, Rafie-Kolpin et al. 2003, Chen 2006, Donnelly et al. 2013). Activation of HRI independent of heme, has been proposed to be mediated by heat shock proteins HSP90 and HSP70 in response to arsenite-induced oxidative stress, heat shock or osmotic stress (Lu et al. 2001).

Regulation of protein synthesis holds the advantage of enabling an immediate and reversible response upon cellular stress (Holcik et al. 2005). Dephosphorylation of eIF2α is required to terminate the ISR and restore protein synthesis and normal cell function. The dephosphorylation process is mediated by the protein phosphatase 1 complex (PP1) (Pakos-Zebrucka et al. 2016). PP1 activity is regulated by GADD34, whose expression is induced as part of the ISR, or by the constitutive repressor of eIF2α phosphorylation (CReP) responsible for targeting PP1 to

eIF2α (Figure 4). CReP acts to sustain translational homeostasis under normal conditions by maintaining low levels of p-eIF2α (Jousse et al. 2003). In contrast, the GADD34-eIF2α pathway acts as an important negative feedback loop to significantly increase eIF2α dephosphorylation once the stress is resolved (Kojima et al. 2003). The dephosphorylation step is important to facilitate translation of accumulated stress-responsive genes and allow for the expression of death-inducing proteins when cellular homeostasis cannot be restored (Pakos-Zebrucka et al. 2016).

While regulation of translation provides the cell with the plasticity needed to respond rapidly, regulation at the transcription level fine-tunes the long-term cellular response through sophisticated gene expression programs. A key output of p-eIF2α is enhanced translation of bZIP (basic leucine zipper domain) transcription factors such as ATF4 (Figure 4). ATF4 belongs to the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB family) and is the best characterized effector of the ISR tailoring the responses to the different cellular stresses (Ameri et al. 2008). Its interaction with other proteins, via the bZIP domain, modulates the transcriptional selectivity of ATF4. Increased levels of ATF4 serve as a transcriptional inducer of numerous genes involved in resolving the cellular stress, such as antioxidant response, amino acid metabolism and transport, protein synthesis, and autophagy. Additionally, ATF4 participates in the GADD34-eIF2α feedback loop by increasing GADD34 transcription, and upregulates genes involved in programmed cell death upon prolonged stress activation (Pakos-Zebrucka et al. 2016, Hetz et al. 2020).

The antioxidant response

It is generally accepted that biological redox reactions promote both physiological responses and pathological cues (Espinosa-Diez et al. 2015, Wen et al. 2020). Low or moderate ROS or reactive nitrogen species act as part of signaling pathways and defense mechanisms initiating homeostatic responses. In contrast, overproduction of such reactive species is detrimental to cells, incurring damage to proteins, DNA, and lipids. In the defense system, cells have evolved enzymatic and non-enzymatic antioxidants and antioxidant signaling pathways to manage scavenging of excessive ROS (Wen et al. 2020).

ROS alters protein function mainly by oxidation of redox-reactive cysteine residues, forming reactive sulfenic acid (-SOH). This oxidized form can react with other nearby cysteines forming disulfide bonds or undergo further oxidation. Most of these redox modifications are reversible by antioxidant reducing systems, enabling ROS regulation of signaling pathways (Ray et al. 2012). Antioxidant molecules can react with oxidants giving them one or two electrons. Glutathione (GSH) is considered the most abundant among the endogenous antioxidants, enabling electron donation as two electron-donating GSH molecules form oxidized GSSG (Lu 2009), as illustrated in Figure 4. GSH acts as a ROS scavenger by direct interaction with reactive species and indirectly by revitalizing other antioxidants systems. As such, GSH homeostasis, regulated by de novo synthesis, utilization, recycling and cellular export, is important for the cell to maintain cellular redox balance (Lu 2009, Espinosa-Diez et al. 2015). GSH is synthesized in two steps; first, glutamate-cysteine ligase catalyzes the formation of γ-glutamylcysteine from L-glutamate and cysteine; second, glutathione synthetase catalyzes the addition of glycine to the C-terminal of γ-glutamylcysteine (Meister et al. 1983, Lu 2009). Under homeostatic conditions, the GSH synthesis rate is largely determined by the cysteine availability and the glutamate-cysteine ligase activity. Cysteine readily autoxidizes to cystine in the extracellular fluid, however once it enters the cell, cystine is rapidly reduced to cysteine (Bannai et al. 1986). Therefore, cystine import through system X_C^- is a crucial factor regulating intracellular levels of cysteine (Bannai et al. 1980, Makowske et al. 1982, Takada et al. 1984, Bannai 1986). System X_C^- is a cystine/glutamate antiporter (Bannai 1986) composed of two components, the catalytic subunit SLC7A11 and the anchoring protein SLC3A2 (Sato et al. 1999). Inhibition of cystine import via system X_C^- trigger ferroptosis (Dixon et al. 2012), an iron-mediated lipid-peroxidation-dependent form of regulated cell death, demonstrating the antiporter's significance in maintaining cellular GSH levels and redox balance (Dixon et al. 2012, Xie et al. 2016, Stockwell et al. 2017) (Figure 4).

Multiple ROS sensing pathways converge on transcription factors, such as Nrf2 (Itoh et al. 1997), AP-1 (activator protein-1) (Sen et al. 1996), and NF-kB (nuclear factor kappa B) (Gloire et al. 2006), to increase the expression of genes involved in ROS detoxification and cellular homeostasis. A major regulator of cytoprotective responses to oxidative stress is the Nrf2-Keap1 (Kelch-like ECH-associated protein 1) pathway (Motohashi et al. 2004, Baird et al. 2011). The half-life and hence transcription factor activity of Nrf2 is tightly regulated by its interaction with Keap1, as illustrated in Figure 4. Keap1 targets Nrf2 for ubiquitination and subsequent degradation by a Cullin-3-mediated ubiquitination complex (Cullinan et al. 2004).

The Keap1-mediated rapid turnover of Nrf2, keeps Nrf2 at low levels under normal conditions. However, when cells are exposed to oxidative stress, oxidation of reactive cysteine residues in Keap1 inactivates Keap1 resulting in Nrf2 stabilization and translocation to the nucleus (Itoh et al. 1999). The oxidation sensitive cysteine residues act as molecular switches that enable Keap1 to regulate steady-state levels of Nrf2 in response to perturbations in the intracellular redox environment (Baird et al. 2011). In the nucleus, Nrf2 heterodimerize with members of the sMaf protein family and targets a *cis*-acting antioxidant response element (ARE) sequence located in the promoter region of many cytoprotective genes (Itoh et al. 1997). Nrf2 triggers the transcriptional upregulation of enzymes involved in antioxidant response such as glutamate-cysteine ligase (rate-limiting step in GSH synthesis), glutathione S-transferases (conjugation of GSH to electrophiles), heme oxygenase-1 (breakdown of heme into the antioxidant biliverdin), system X_C^- (cystine/glutamate antiporter), as well as genes involved in lipid metabolism, NADPH regeneration and autophagy biogenesis (Motohashi et al. 2004, Baird et al. 2011, Espinosa-Diez et al. 2015).

Autophagy

One of the key pathways that mediate stress-induced metabolic adaptation to prevent potential damage is autophagy. Autophagy, which translates to *self-eating*, is a highly conserved catabolic process delivering intracellular material to the lysosomes for degradation and recycling of building blocks (Mizushima et al. 2011). The autophagic process is thus able to remove damaged organelles and protein aggregates, and provide the cell with nutrients for vital cellular functions. This makes autophagy both an important housekeeping pathway under homeostatic conditions, and cytoprotective pathway under stress.

The autophagy pathway is generally subdivided into three categories; microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA) (Boya et al. 2013). The division is based on the mechanism of delivering cytoplasmic material to the lysosome, which for microautophagy involves the direct invagination of the lysosomal membrane (Oku et al. 2018) and for CMA involves a transmembrane protein translocation complex in the limiting membrane of the lysosome (Kaushik et al. 2018). By contrast, during macroautophagy the formation of a double-membrane vesicle, the autophagosome, engulfs the cytoplasmic cargo

and fuses with the lysosome (Klionsky et al. 1999, Yang et al. 2010, Mizushima et al. 2011). Macroautophagy is the best characterized of the three and the focus of this thesis, and will henceforth be referred to as autophagy. Autophagy was initially believed to be non-selective, sequestering an arbitrary portion of the cytoplasm (Kopitz et al. 1990, Seglen et al. 1990). Today, we know that autophagy can also be selective (Beese et al. 2020), where specific cargo receptors serve as adaptors to target substrate such as ER or mitochondria (Klionsky et al. 2007). This work has, however, focused on the non-selective phagophore-mediated bulk autophagy.

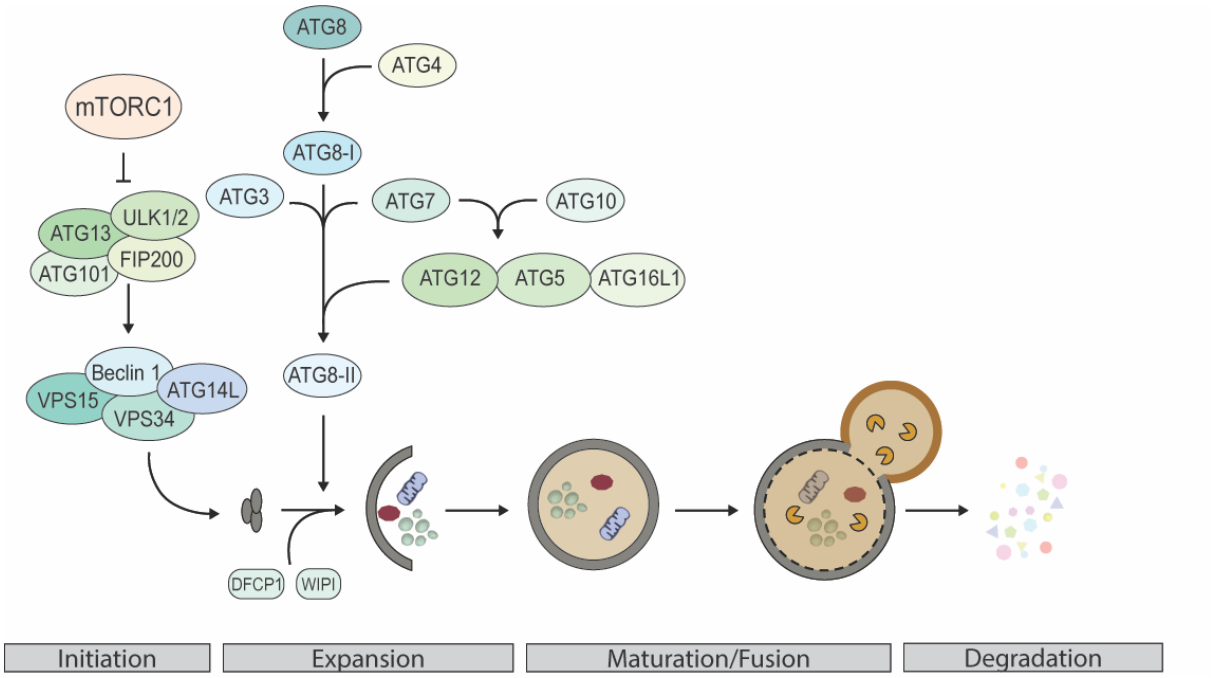

Figure 5. The autophagy pathway is initiated by the ULK complex (ULK1 and 2, ATG13, ATG101, FIP200), which translocate to the phagophore assembly site and engage phospholipid synthesis through the PI3KC3 complex (Vps34, Vps15, Beclin 1, ATG14L). DFCP1 and WIPI proteins binds the resulting PI(3)P, and recruits the E1-, E2- and E3-like conjugation machinery (ATG3, ATG7, ATG10, ATG12, ATG5, ATG16L1) in the proximity to the nascent phagophore. Cytosolic ATG8-I is conjugated to the phagophore after cleavage by ATG4, forming the lipidated ATG8-II. The phagophore expands and sequesters cytosolic components and organelles, and ultimately closes in on itself to from the autophagosome. Upon fusion with the lysosome, the inner autophagosomal membrane and the sequestered cargo is degraded by lysosomal hydrolases.

Genes encoding proteins involved in autophagy were first described in yeast (Tsukada et al. 1993), however several independent groups identified genes essential for autophagy during the same period, resulting in several names for the same gene/protein and the need for a common

nomenclature for autophagy-related (ATG) genes/proteins (Klionsky et al. 2003). Mechanistically the autophagy pathway involves a complex set of steps depending on the sequential action of a distinct set of ATG proteins (Itakura et al. 2010), as illustrated in Figure 5. A subset of 'core' ATG proteins are essential for autophagosome formation. This core molecular machinery consists of: (1) Atg1/the unc-51-like kinase 1 (ULK1) complex (ULK1 and 2, ATG13, FIP200, ATG101), (2) the class III Phosphatidylinositol 3-kinase (PI3KC3) complex (Vps34, Vps15, Beclin 1, ATG14L), (3) two ubiquitin-like protein conjugation systems (ATG12, ATG5, ATG16L1 and Atg8/LC3/GABARAP), and (4) two multi-spanning transmembrane proteins (ATG9L and VMP1) (Kabeya et al. 2000, Yang et al. 2010, Mizushima et al. 2011).

Autophagy is initiated by the concerted action of the ULK1 complex and PI3KC3 complex, acting downstream of the target of rapamycin (mTOR) complex 1 (mTORC1) (Chan et al. 2007, Ganley et al. 2009, Hosokawa et al. 2009, Mizushima et al. 2011, Russell et al. 2013). Under nutrient-rich conditions, mTORC1 keeps the ULK1 complex in an inactive state by phosphorylating ULK1 at Serine757 (Kim et al. 2011) and ATG13 at Serine258 (Puente et al. 2016). Upon nutrient starvation or pharmacological inhibition of mTORC1, mTORC1 dissociates from the ULK1 complex, resulting in dephosphorylation and translocation of the complex to the site of phagophore assembly (Ganley et al. 2009, Kim et al. 2011, Puente et al. 2016). Assembly of the initial phagophore membrane, as a cup-shaped structure in the ER, called omegasome, requires PI3KC3 activation (Axe et al. 2008, Hayashi-Nishino et al. 2009). The activated ULK1 kinase is recruited to the PI3KC3 complex through binding of the ATG14L component (Matsunaga et al. 2010). ULK1 thus phosphorylates Beclin 1 activating the Vps34 kinase (Russell et al. 2013). This leads to generation of phosphatidylinositol 3-phosphate (PI(3)P) at the phagophore membrane by phosphorylation of the lipid phosphatidylinositol, recruiting the machinery involved in phagophore expansion (Volinia et al. 1995, Russell et al. 2013).

PI(3)P serves as a binding site to recruit specific effectors such as DFCP1 (zinc finger FYVE domain-containing protein 1) (Derubeis et al. 2000, Axe et al. 2008) and WIPI (WD-repeat protein-interacting phosphoinositide) (Proikas-Cezanne et al. 2004, Mauthe et al. 2011). These effectors rapidly recruit the ATG12, ATG5, ATG16L1 ubiquitin-like conjugation system. In an E1- and E2-like manner (facilitated by ATG7 and ATG10), ATG5 and ATG12 are conjugated and non-covalently associated with ATG16L1, which binds WIPI in the forming phagophore (Itakura et al. 2010, Dooley et al. 2014). This complex further acts as an E3-like

enzyme for LC3 lipidation, who's ubiquitin-like reactions are occurring in parallel. There are two subfamilies of ATG8 proteins in mammals; the LC3 subfamily of LC3A, LC3B and LC3C and the gamma-aminobutyric acid receptor-associated protein (GABARAP) subfamily of GABARAP, GABARAPL1 and GABARAPL2/Golgi-associated ATPase enhancer of 16 kDa (GATE-16) protein (Shpilka et al. 2011). Newly synthesized pro-LC3 is cleaved at the C-terminal by the cysteine protease ATG4 exposing a glycine and generating the cytosolic LC3-I (Kirisako et al. 2000). This glycine residue is used to conjugate LC3-I to phosphatidylethanolamine (PE) in the phagophore membrane in an E1- and E2-like manner facilitated by ATG3 and ATG7, forming the lipidated LC3-PE (LC3-II) (Ichimura et al. 2000, Fujita et al. 2008). The 'ATG12, ATG5, ATG16L1'- E3-like enzyme recruits and stimulates the E2-like protein ATG3, and hence promote LC3 lipidation (Hanada et al. 2007). LC3-II attaches to both faces of the phagophore membrane, and has been proposed to promote autophagosome formation (Nakatogawa et al. 2007, Weidberg et al. 2010). Furthermore, the ATG8s recruit cargo receptors for selective degradation by binding to the LIR (LC3-interacting region) motif of the cargo receptors (Pankiv et al. 2007, Noda et al. 2008). The forming autophagosome can thus capture cargo either in bulk or in a highly selective manner, emphasizing the importance of the pathway in relation to various cellular stress (Reggiori et al. 2012). ATG4 is further involved in deconjugating and hence removing LC3 from the outer surface of closed autophagosomes (Satoo et al. 2009).

The completed autophagosomes are then transported by motor proteins along the cytoskeleton to the perinuclear region in proximity to lysosomes (Cardoso et al. 2009, Pankiv et al. 2010, Maday et al. 2012). The molecular mechanisms of transport and fusion is incompletely understood, but known to involve RAB GTPases (Ganley et al. 2011), membrane tethering factors (McEwan et al. 2015, Wartosch et al. 2015), and SNARE proteins (Fader et al. 2009, Itakura et al. 2012). During maturation the autophagosome may fuse with endosomes forming an amphisome (Gordon et al. 1988, Berg et al. 1998), which subsequently fuses with the lysosome, or the autophagosome may directly fuse with the lysosome (Dunn 1990). Upon fusion with the lysosome, the inner autophagosomal membrane and the sequestered cargo is degraded by lysosomal hydrolases (Mizushima et al. 2002).

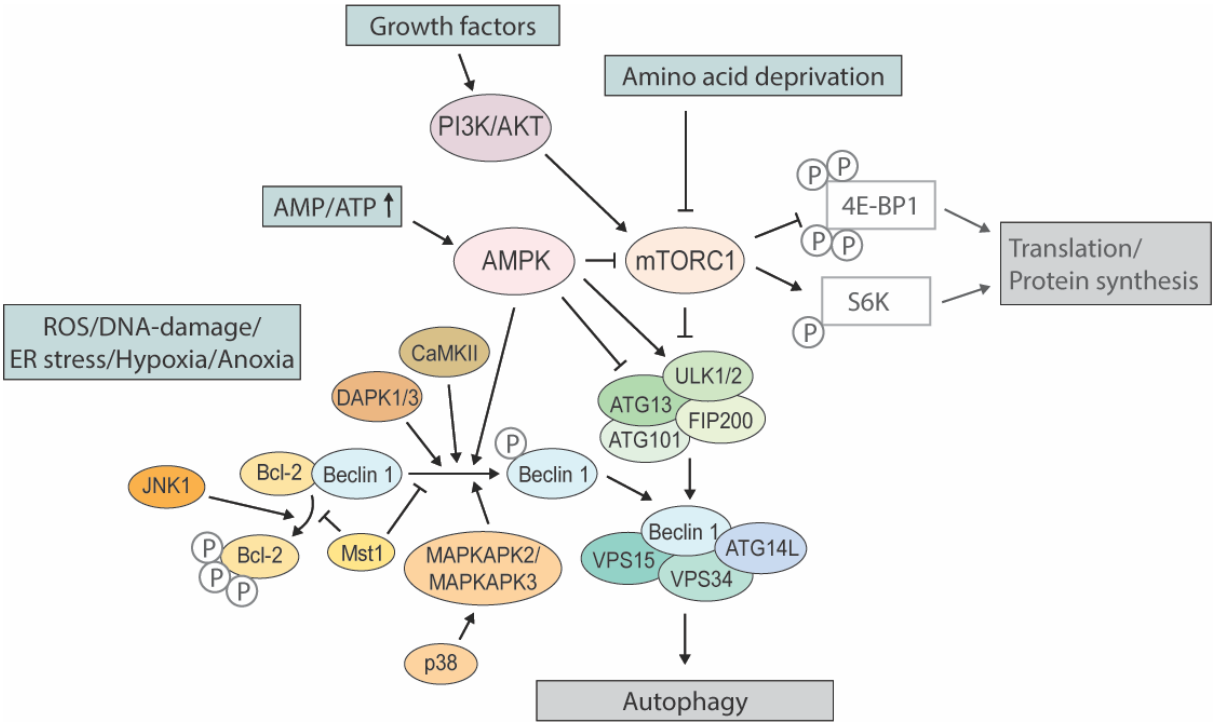

Figure 6. mTORC1 is the master negative regulator of autophagy. Activated mTORC1 will phosphorylate the translation inhibitor 4E-BP1 and ribosomal protein S6 kinase leading to translation initiation and protein synthesis, while at the same time inactivate autophagy. Deprivation of amino acids inhibits mTORC1, thus activating autophagy. Growth factor signaling through PI3K/AKT pathway inhibits autophagy by activating mTORC1. The energy-sensor AMPK may activate autophagy by inhibiting mTORC1 or directly phosphorylating and activating ULK1 or Beclin 1, or suppress autophagy by phosphorylating ATG13. Various stress-inducing factors converge on Beclin 1 for autophagy regulation.

Efficient regulation of autophagy is crucial for the cell to adapt to various stress and autophagosome formation is thus highly inducible upon demand. Being tightly coupled to the cell's metabolic needs, different nutrient-sensing pathways convey signals to the autophagy machinery, as illustrated in Figure 6. The large serine/threonine protein kinase mTOR regulates cellular homeostasis by coordinating anabolic and catabolic processes (Blommaart et al. 1995, Sengupta et al. 2010). Two mTOR complexes exist, wherein mTORC1 is found to be the master negative regulator of autophagy. Activated mTORC1 will phosphorylate the translation inhibitor 4E-BP1 and ribosomal protein S6 kinase leading to translation initiation and protein synthesis. While at the same time binding and phosphorylating ATG13 (Puente et al. 2016) and ULK1 (Kim et al. 2011), thus inactivating autophagy (Kim et al. 2019). Deprivation of amino acids is the best characterized trigger of autophagy, inhibiting mTORC1 releasing the breaks on autophagy (Seglen et al. 1980, Seglen et al. 1984, Blommaart et al. 1995). AMP (5′

adenosine monophosphate)-activated protein kinase (AMPK) is another well characterized regulator of autophagy. AMPK is an energy-sensing kinase activated when intracellular ATP levels are reduced, resulting in a relative increase in AMP or ADP. Thus, activated AMPK initiates catabolic processes such as autophagy to generate more ATP. The best-studied mechanism of autophagy activation by AMPK is by suppression of the mTORC1 pathway (Mihaylova et al. 2011). However, evidence indicates that AMPK can bypass mTORC1 and induce autophagy by directly phosphorylating ULK1 at Serine317 and Serine777 (Kim et al. 2011), or suppress autophagy by phosphorylation of ATG13 at Serine224 (Puente et al. 2016). Oppositely, growth factor signaling through the class I Phosphatidylinositol 3-kinase (PI3K)/AKT pathway is known to inhibit autophagy by activating mTORC1 (He et al. 2009). Moreover, several stress-inducing factors such as ROS, DNA-damage, hypoxia/anoxia, and ER-stress are also described to fine-tune autophagy through activation of stress-responsive kinases (He et al. 2009), and upregulation of transcription factors, like ATF4, TFEB and TFE3 (Füllgrabe et al. 2016).

Several of the stress-activated signaling pathways have been demonstrated to converge on Beclin 1 for autophagy regulation (Menon et al. 2018). Beclin 1 was originally identified as an interaction partner of the anti-apoptotic protein Bcl-2 (Liang et al. 1998), and binding of Bcl-2 to the N-terminal Bcl-2 homology (BH3) domain on Beclin 1 is found to inhibit autophagy (Pattingre et al. 2005). Death-associated protein kinase-1 (DAPK1) phosphorylates the BH3-domain of Beclin 1, disturbing its interaction with Bcl-2 facilitating autophagy (Zalckvar et al. 2009). The proapoptotic kinase Mst1 (mammalian STE20-like protein kinase 1) is on the other hand found to stabilize the Beclin 1-Bcl-2 interaction, inhibiting autophagy in cardiomyocytes (Maejima et al. 2013). The stress-activated mitogen-activated protein kinases (MAPKs), c-jun NH_2-terminal kinase (JNKs) and p38 MAPKs, are found to increase the lipid kinase activity of the Vps34 in PI3KC3 autophagy nucleation complex through its effects on Bcl-2 and Beclin 1 (Wei et al. 2015). JNK1 was demonstrated to phosphorylate Bcl-2 inducing the dissociation of the Beclin 1-Bcl-2 complex, while the p38-downstream kinases MAPKAPK2 and MAPKAPK3 directly phosphorylate Beclin 1 at the Serine 90 residue (Wei et al. 2015). The Serine 90 and 93 phosphorylation sites of Beclin 1 was originally identified during starvation-induced autophagy (Fogel et al. 2013), but have later been demonstrated to be a target of several kinases such as AMPK (Kim et al. 2013), DAPK3 (Fujiwara et al. 2016), and CaMKII (Calcium/calmodulin-dependent protein kinase II) (Li et al. 2017).

AIMS OF THE STUDIES

Fundamental studies on how NPs interact with biological systems is imperative for their use as therapeutic or diagnostic agents. The three papers of this thesis investigate how NPs affect cellular stress responses *in vitro* (in cultured cells) with the aim to characterize the stress response pathways and decipher the consequences such stress impose on cytotoxicity, cell death mechanisms and the lysosomal intracellular degradation pathway, autophagy.

To exploit the potential of NPs in medicine, detailed knowledge on the nature and extent of NP-induced cellular stress responses is required. In the work of **Paper I**, we aimed to study how minor alterations in the NP polymer composition can affect the type and magnitude of cellular stress, and whether this reflects NP-induced cytotoxicity and mode of cell death.

Nanoparticles trigger a variety of cellular stress responses, among which autophagy is one of the pathways important to maintain cellular homeostasis. By using the highly similar PACA particles employed in Paper I, we aimed in the work of **Paper II** to systematically study how the PACA variants affects the autophagy pathway and whether the PACA-induced stress responses, identified in Paper I, relate to the effect on autophagy.

Our investigations in Paper II exemplify the ambiguity that may be associated with LC3 as a marker of autophagy regulation upon NP exposure. In the work of **Paper III**, we aimed to investigate how NPs may regulate autophagic cargo degradation. This was performed by comparing several functional autophagy assays, and we used the PBCA particle, one of the PACA NPs employed in Paper I and II, as a model particle. Both because this NP is known to induce redox imbalance (Paper I), which is a representative feature of several NPs, but also to further explore the surprising block in LC3 lipidation that we observed in Paper II.

SUMMARY OF PAPER I

Small variations in nanoparticle structure dictate differential cellular stress responses and mode of cell death

NPs can be highly reactive structures and induce cellular stress upon treatment. In the aim to regain homeostasis the cell can initiate a variety of stress responses. However, if the insult is too severe the originally induced pro-survival pathways may be switched to a pro-death mechanism. Consequently, the NP-induced stress responses may contribute to the therapeutic response, but may also counteract the intended therapy.

To investigate how minor differences in NP composition may affect the outcome of NP-cell interaction, we employed PACA NPs made from alkylcyanoacrylate monomers differing only in their alkyl side chains. The resulting particles, PBCA (butyl), PEBCA (ethylbutyl) and POCA (octyl), exhibited similar physicochemical characteristics, such as size (133-153 nm), polydispersity (0.13-0.16) and surface charge (-2.4 to -3.4 mV).

Strikingly, the highly similar PACA particles displayed different cytotoxicity in a panel of cell lines. In general, POCA induced the highest cytotoxicity, whereas PEBCA was the least toxic. All PACAs activated the ISR observed by phosphorylation of eIF2α in a manner dependent on several ISR kinases, however with a stronger dependency on the HRI kinase for PBCA. The POCA particle induced ER stress through PERK and IRE1 activation, and selectively induced apoptotic cell death attributed to caspase activation and fully rescued by the pan-caspase inhibitor z-VAD. The PEBCA and PBCA particles induced oxidative stress, accumulating ATF4 and Nrf2, and induced the cell death mechanism *ferroptosis* in a manner dependent on the cell's ability to mount a redox defense. Consequently, the PEBCA- and PBCA-induced cytotoxicity was demonstrated to be highly dependent on the level of cystine and transcription of the cystine transporter *SLC7A11*. PEBCA induced pro-survival Nrf2 activation to a larger extent than PBCA, upregulating SLC7A11 among other antioxidant proteins. The PEBCA- and PBCA-induced ATF4 and Nrf2 accumulation protected the cells against ferroptosis, therefore we concluded that the increased ability to activate a prolonged cell protective response contributes to the lower cytotoxicity of PEBCA particles.

In conclusion, our study demonstrates that even minor alterations in PACA NP composition have consequential effects on cellular responses and mode of cell death. It further underscores the importance of detailed investigations of the NP-cell interaction for optimal exploitation of NPs in medicine.

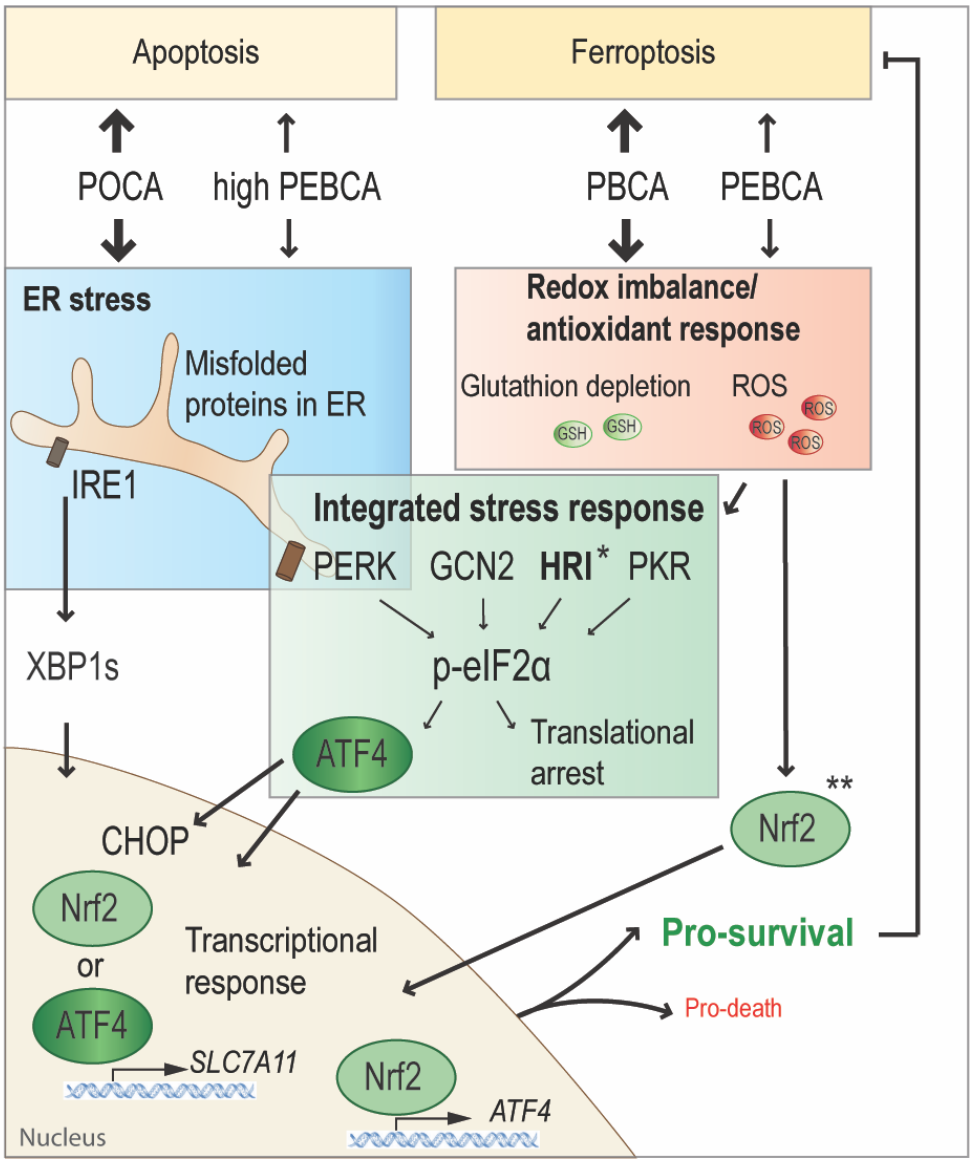

Figure 7. Summarized illustration of Paper I**.** A model of the variation in PACA-induced cellular stress responses and mode of cell death. * HRI is particularly induced by PBCA. ** Nrf2 is particularly induced by PEBCA.

SUMMARY OF PAPER II

Structural Variants of poly(alkylcyanoacrylate) Nanoparticles Differentially Affect LC3 and Autophagic Cargo Degradation.

In **Paper I**, we showed that small variations in the alkyl side chains of PACA NPs, butyl (PBCA), ethylbutyl (PEBCA), or octyl (POCA), differentially induce ER stress and redox imbalance in human cell lines. The stress induced by NPs may initiate a concerted action of stress response pathways. One of the key pathways that mediates stress-induced adaptation is autophagy, having fundamental functions in cellular homeostasis and survival. Understanding the details of how NPs influence autophagy-related proteins and the autophagic process is thus of great interest.

Here, we systematically investigate how the PACA variants studied in Paper I, affect the autophagy pathway and whether the PACA-induced stress responses relate to the effect on autophagy.

Interestingly, we found that treatment with both PEBCA and POCA particles led to intracellular accumulation of the autophagosome marker LC3-II. PEBCA increased transcription of *LC3B* mRNA by activation of the ISR and accumulation of ATF4, causing elevated LC3-II protein levels. POCA, on the other hand, increased LC3-II protein levels as a consequence of inhibited LC3-II degradation determined both by inhibition in LC3 flux and autophagic cargo degradation (LLPD). In contrast, treatment with PBCA did not affect LC3-II protein levels, even though activation of the ISR and accumulation of ATF4 increased LC3B mRNA levels. On the contrary, PBCA seemed to inhibit LC3-lipidation, reducing LC3 flux and autophagic LLPD. Moreover, lysosomal dysfunction did not attribute the different effects on autophagy by the PACA NPs.

In conclusion, our data demonstrate that PACA NP composition have profoundly different impact on the autophagy-related gene product LC3 and autophagic degradation, being a key process for cellular homeostasis. These findings have important implications for the choice of PACA monomer in different therapeutic settings. Additionally, our data highlights that careful monitoring of autophagic flux and cargo degradation is critical for drawing accurate conclusions on the impact of NP treatment on autophagy.

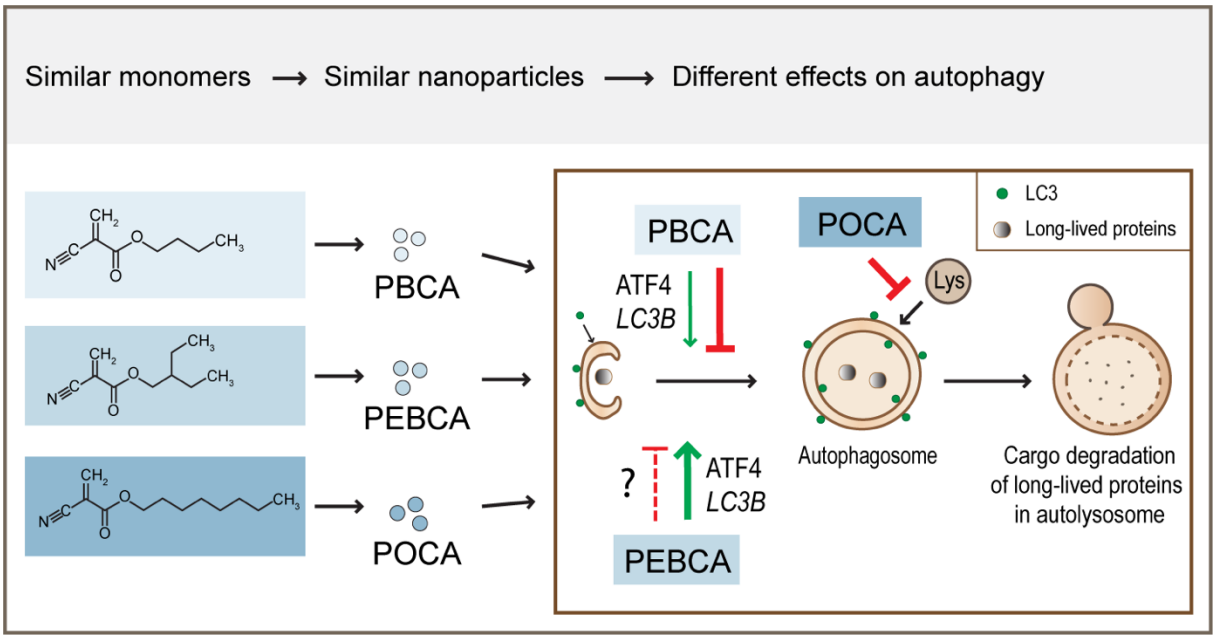

Figure 8. Summarized illustration of Paper II. A simplified model of the PACA NPs' differential effects on LC3B and the various steps of autophagy. Lys: Lysosomes.

SUMMARY OF PAPER III

Perturbation of cellular redox homeostasis dictates divergent effects of polybutyl cyanoacrylate (PBCA) nanoparticles on autophagy.

Cells undergo rapid changes to adapt their metabolism and protect themselves against potential damage. Autophagy is a major cytoprotective pathway in this respect, and efficient regulation is thus crucial for the cell. Oxidative stress is both recognized as a regulator of autophagic activity and a frequent underlying mechanism of NP-induced stress. In Paper II we demonstrated that the highly similar PACA NPs had very different effects on LC3 levels. Where POCA and PEBCA increased LC3-II, treatment with PBCA surprisingly reduced LC3-II levels due to a block in LC3 lipidation to the forming autophagosome.

Intrigued by this, we performed a comprehensive investigation into how the PBCA particle affects the different steps of autophagy, from LC3 lipidation and autophagosome formation, to degradation of typical autophagic cargos in several functional assays.

Strikingly, PBCA induced opposing effects on autophagy regulation depending on the NP concentration. Treatment with the highest concentration of PBCA (25 µg/ml), inhibited Torin1-induced LC3 lipidation and autophagic cargo degradation in a redox imbalance-dependent manner. The inhibition was fully reversible by co-treatment with antioxidants and was mimicked by H_2O_2. In contrast, at lower concentrations (<12.5 µg/ml) PBCA increased autophagic degradation beyond that induced by the mTORC1-inhibitor Torin1, without a concomitant increase in LC3 turnover. The stimulatory effect was found to depend on activation of both the JNK and p38-MAPKAPK2 pathway downstream of redox stress, which led to increased phosphorylation of Bcl-2 and the Ser90 residue of Beclin 1, respectively.

In conclusion, our data demonstrates how the degree of redox imbalance induced by PBCA can modulate the autophagic response. At moderate levels, the cell can respond by stimulating autophagy to regain homeostasis. In contrast, if the oxidative imbalance is too severe, the autophagic process is inhibited, and cell survival decrease over time. Furthermore, this study illustrates the high grade of corroboration between several functional autophagy assays.

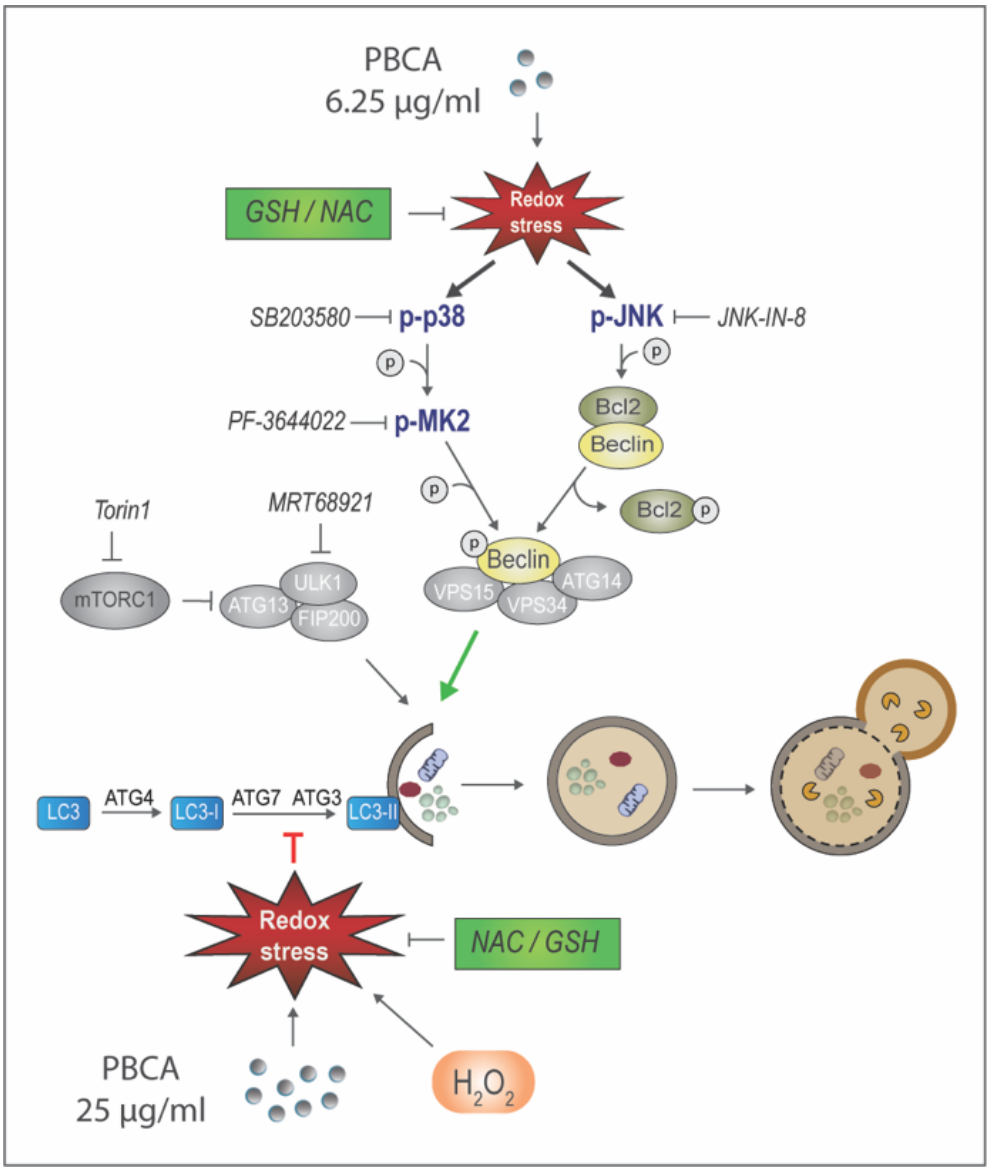

Figure 9. Summarized illustration of Paper III. A model of the dual effect on autophagy depending on the severity of the PBCA-induced oxidative stress.

EXPERIMENTAL CONSIDERATIONS

All methods used in this work is thoroughly described in their respective papers. This section discusses important methodological considerations with regards to the primary methods employed throughout this thesis.

Aspects of nanoparticle characterization and treatment

Fundamental to NP studies is proper characterization of their physicochemical properties as they provide the foundation for systematic investigations and are important determinants of NP cytotoxicity. The PACA NPs used in this thesis were obtained through collaboration with Dr. Yrr Mørch at SINTEF (SINTEF Industry, Dept. Biotechnology and Nanomedicine, Trondheim, Norway). SINTEF have the proper equipment, staff and expertise for both manufacturing and characterizing NPs. The PACA NPs are synthesized by mini-emulsion polymerization using cyanoacrylate monomers having different alkyl groups; butyl (PBCA), ethylbutyl (PEBCA) and octyl (POCA) as illustrated in Figure 10. The mini-emulsion polymerization is a one-step method mixing the oil phase with the aqueous phase using an ultrasonic homogenizer. The oil phase consists of the specific PACA monomer and the co-stabilizer miglyol®810N (2 wt%, Cremer). When preparing NPs for cellular uptake studies, also the hydrophobic dye pentamer hydrogen thiophene acetic acid methyl ester (pHTAM, 0.5 wt%) was included in the oil phase. The aqueous phase consists of hydrochloric acid (HCl, 0.1 M) and the PEG-surfactants Kolliphor® HS15 (6 mM) and Pluronic® F68 (long PEG), 1mM). The anionic polymerization was initiated by addition of the PEG-surfactants, and the polymerization reaction was allowed to carry out overnight. After which, unreacted monomers and surplus of surfactants were rinsed by extensive dialysis. The final concentration of "free" surfactant in the NP dispersions were found to be far below cytotoxicity levels. The PACA NPs were further characterized at their facilities by size, size distribution and surface charge in 0.01 M phosphate buffer by a Zetasizer Nano ZS (Malvern Instruments) using dynamic and electrophoretic light scattering. They found the PACA NPs to be stable for many years when kept at acidic conditions (< pH 5) at 4 °C. This extraordinary stability was highly beneficial and simplified our use of these NPs, and importantly, data obtained during a single project resulted from the use of just a few (Paper I and II) or in fact only one batch of NPs (Paper III). Degradation of the PACAs increased at pH

6-7 (Sulheim et al. 2016). Moreover, the PACA NPs were found to be colloidally stable when transferred to either PBS, cell medium or human plasma at physiological pH. However, when degradation starts the NPs swell due to hydrolyzation and partly agglomeration. This is a beneficial property with regards to drug delivery as the NPs eventually need to degrade in order to release the payload and be excreted from the body.

By the mini-emulsion method the dye/drug is associated with the PACA NP through hydrophobic interactions, not covalently bound, and is hence a constituent of the oil phase. This makes the dye/drug susceptible to leakage once in contact with other hydrophobic domains (Klymchenko et al. 2012, Snipstad et al. 2017). It has been demonstrated that the less hydrophobic dye Nile Red is delivered directly to the cytosol by contact-mediated transfer from the PBCA NP, not requiring endocytosis for internalization. Whereas the more hydrophobic dye pHTAM, which was included in the particles used in Paper I, requires endocytosis to be internalized and is hence internalized with the PBCA NP (Snipstad et al. 2014). This is an important aspect, as fluorescence from released dye can wrongly be interpreted to be associated with the NP. Such a contact-mediated transfer and leakage from various NPs has been demonstrated by several groups (Xu et al. 2009, Andreozzi et al. 2013, Snipstad et al. 2014, Simonsson et al. 2016).

Upon reception in our lab, the PACA NPs were stored as recommended at 4 °C. Additionally, before the NPs were employed in any experiment, we validated the physicochemical characterization, such as size and size distribution.

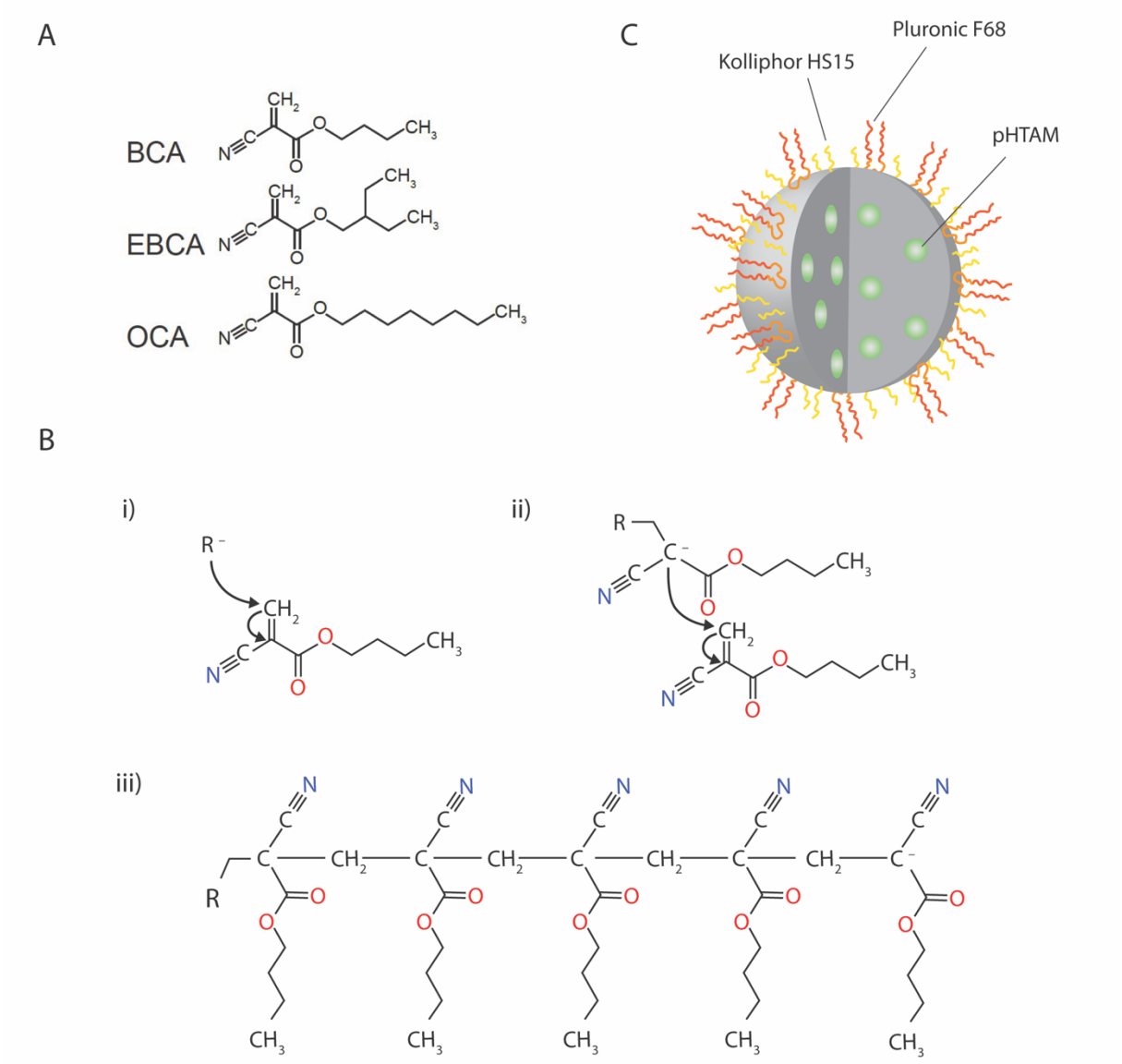

Figure 10. The PACA NPs used in this work were synthesized by mini-emulsion polymerization using the monomers butyl cyanoacrylate (BCA), ethylbutyl cyanoacrylate (EBCA) and octyl cyanoacrylate (OCA) (**A**). The anionic polymerization process of BCA **(B)**: a group containing a free electron bind the CH_2 group, and the free electron is moved to the central C-atom, i). The now negative charged BCA can act on a neighboring BCA molecule, ii). The chain reaction leads to the polymerization of the PBCA molecules, iii). The completed PACA NP with the dye, pHTAM, associated through hydrophobic interactions and the PEG-polymers, Kolliphor HS15 and Pluronic F68, chemically bound to the PACA polymer (**C**).

Our three papers investigate how NPs affect cellular stress responses *in vitro* (in cultured cells) wherein there are several aspects to consider. First, the colloidal NP suspension was vortexed directly before use to ensure full dispersion in the solvent. Second, we aimed to keep the NP-to-cell ratio as constant as possible throughout the different assays, as this parameter has proved

to be more important than NP concentration when working with cells seeded in tissue culture plates (Unciti-Broceta et al. 2015, Drasler et al. 2017, Moore et al. 2019). To obtain a constant NP-to-cell ratio when working with tissue culture dishes ranging from 96-well plates to 6-well plates, the volume of growth medium in the dish during NP treatment was adjusted according to the different well areas. Third, to be able to compare NP cytotoxicity across our panel of cancer cell lines, all cell types were seeded to have a confluency of approximately 80% at the time of NP treatment. Fourth, the protein corona forming around NPs in biological systems is an important topic in NP cytotoxicity as it dictates the NP-cell interaction (Nel et al. 2009, Corbo et al. 2016, Wang et al. 2019). To keep the protein corona as similar as possible between amino acid starvation conditions (EBSS, Earle's balanced salt solution) and complete medium we supplemented the EBSS with dialyzed, and thus amino acid free, fetal bovine serum. By using a dialysis tube cutoff of 3.5 kDa, we exclusively remove amino acids and other small soluble components while maintaining the serum proteins.

Assessment of cellular effects of nanoparticles

Assessment of NP cytotoxicity generally fall into two categories, functional assays (addressing the effects of NPs on cellular processes) and viability assays (addressing cell death or metabolic changes induced by NP treatment). Verifying that the three PACA NPs were internalized to the same degree across a panel of cell lines, was critical to this work to be able to relate the differences in cellular responses and cytotoxicity between the PACA variants, and between different cell lines, to the actual amounts of NPs that were internalized. Throughout this work we have mainly assessed effects of subtoxic concentrations of NPs, giving less than 20% reduction in the cellular ATP levels (as a surrogate for viability) after 24 hours and no reduction after 4 hours, to be able to evaluate effects of NP treatment on living cells.

Viability assays

Cytotoxicity assays are normally based on assessing damage to cellular membranes, cell viability/metabolic activity or cell proliferation. We have combined various cytotoxicity assays to reveal specific information regarding the mechanism of cell death by the PACA variants. The reagent CellTox™ Green (Promega) is based on a fluorescent DNA binding dye that cannot

enter intact live cells, but binds DNA when accessible upon loss of membrane integrity. Fluorescence only occurs upon cell death correlating with the NP-induced cytotoxicity. In a similar manner, we also used the DNA-binding dyes propidium iodide or Hoechst.

By combining assays monitoring cell integrity with assays monitoring caspase activity, such as CellEvent™ Caspase-3/7 (Invitrogen), we can determine whether cell death is caused by apoptosis. CellEvent™ Caspase-3/7 Green Detection Reagent is a four-amino acid peptide conjugated to a nucleic acid-binding dye, inhibiting the ability of the dye to bind DNA. Activation of caspase-3 is an essential event during apoptosis (Porter et al. 1999), and after caspase-3/7 activation the four-amino acid peptide is cleaved off, enabling the dye to bind DNA. If Caspase-3/7 fluorescence precedes membrane integrity staining during NP treatment, we can infer that the NPs induce cell death by apoptosis.

In contrast to cell death assays, which estimate the accumulation of dead cells, viability assays provide information on cell viability, often based on the level of metabolic activity. However, viability assays offer little information about the underlying mechanism for the observed changes in metabolic activity. An observed reduction may be caused by a reduced cell number due to an anti-proliferative effect of the treatment, or alternatively, cellular metabolism is reduced without alterations in cell number. The assay CellTiter-Glo® (Promega) determines the number of viable cells based on quantification of cellular ATP levels, thus determining the amount of metabolically active cells.

Functional assays

Throughout the three papers of this thesis, we combined various methods to address the cellular stress response pathways affected by the PACA NPs. In Paper I and II this was deciphered mainly using protein markers, pharmacological inhibitors, and siRNA-mediated depletion of proteins (see next section). In Paper III, we turned our focus to measuring functional autophagic degradation after NP treatment. Through a comprehensive set of assays, we monitored the dynamic flux through the entire system, including fusion with lysosomes and processing within the lysosomes.

LC3 flux assay

The formation of LC3-II has been recognized as a hallmark of autophagy regulation, since it was demonstrated that LC3 translocate from cytosol (LC3-I) to the phagophore (LC3-II) upon

starvation (Kabeya et al. 2000). Immunoblotting provides a simple way of evaluating changes in LC3 as LC3-II migrates faster than LC3-I during gel electrophoresis. This results in bands of approximately 14 and 16 kDa, respectively, that can be simultaneously detected using an antibody against endogenous LC3. However, LC3-I and LC3-II can also be distinguished by fluorescence microscopy using antibodies or expression of fluorescently tagged LC3. Due to the membrane localization of LC3-II it will appear as punctate structures, while LC3-I being cytosolic will give a diffuse staining pattern (Klionsky et al. 2021). Upon increased autophagy initiation there is normally an enhanced conversion of LC3-I to LC3-II, which has led to the common interpretation that increased LC3-II indicates increased functional autophagy. However, it has later been clarified that the presence of the LC3 marker itself does not necessarily reveal the actual function of autophagy. Increased LC3-II may arise as more phagophores and autophagosomes are formed, but also as an accumulation-effect due to inhibited degradation. By assessing 'LC3 flux' in the presence or absence of a lysosomal inhibitor such as Bafilomycin A1 (BafA1), we can distinguish between alterations in LC3 lipidation and LC3 degradation. If phagophore formation is increased, inhibiting degradation by BafA1 will cause an enhanced accumulation of LC3-II compared to the inhibition alone (BafA1). However, if the LC3-II increase is already caused by an inhibition in degradation the addition of BafA1 will not change the accumulated LC3-II level (Mizushima et al. 2010, Klionsky et al. 2021). The results from Paper II and Paper III of this thesis clearly emphasize the need to complement the use of LC3-II as a marker, with measures of overall degradation of autophagic cargo proteins, to interpret how NPs may regulate autophagy. For this we exploited the well-established lactate dehydrogenase (LDH) sequestration assay (Luhr et al. 2018) and long-lived protein degradation (LLPD) assay (Luhr et al. 2018, Klionsky et al. 2021), and further made the effort to establish mKeima-based assays in our lab (Katayama et al. 2011).

LDH sequestration assay

The LDH sequestration assays is a method to quantitatively measure the sequestration rate of bulk cytosolic cargo into closed autophagosomes, by using the cytosolic protein LDH as a marker (Figure 11). LDH is a prominent marker of autophagic sequestration as it is ubiquitously expressed, exclusively degraded by autophagy, and sequestered at the same rate as other cytosolic proteins with varying half-lives (Kopitz et al. 1990). To quantify the net sequestration rate of LDH independent of its degradation, an inhibitor of lysosomal degradation, such as BafA1, needs to be included in the experimental procedure. After a given treatment period, the plasma membrane of cells is disrupted by a short electric pulse leaving the membranes of

organelles and vesicles intact. As such LDH sequestered into complete autophagosomes, unaffected membranes, can be separated from cytosolic LDH by density gradient centrifugation. The amount of LDH confined within closed autophagosomes to the total amount of LDH in each sample is measured by the enzymatic activity and divided by the treatment time with BafA1 giving the rate of sequestration. Measuring the enzymatic activity of LDH provides an amplified readout of the LDH level and makes the assay highly sensitive to compare changes in LDH sequestration rate. A strength of the method is thus that it measures sequestration of endogenous cargo in a quantitative manner. As LDH is a small protein, it leaks out of incompletely sealed phagophores and hence only accumulate when the autophagosome is completely sealed. This further allows for specific assessment of the phagophore closure step and the distinguishment of blocked autophagic degradation prior to or post phagophore closure. In a similar manner to LC3 flux, if LDH sequestration is further enhanced compared to the inhibition (BafA1) alone, it is an indication that macroautophagic sequestration is increased. Conversely, if sequestration is absent in the presence of BafA1, autophagy is inhibited at a step prior to phagophore closure. As the assay relies on inclusion of a lysosomal inhibitor to accumulate the sequestered LDH, and one cannot exclude the possible impact of the inhibitor on the autophagic process, time points should be kept short (typically 2-4 hours). A major advantage with the LDH sequestration assay is that it can be carried out in any mammalian cell line without the need to introduce exogenous probes (such as mKeima), since LDH is expressed in all mammalian tissues. As such, also primary cells having a limited lifespan can be assessed by this method.

Long-lived protein degradation assay

The long-lived protein degradation assay is a pulse-chase radiolabeling assay measuring the degradation of long-lived proteins, as illustrated in Figure 11. In the *pulse* cells are treated with radiolabeled ^{14}C valine which gets incorporated into newly synthesized proteins over a period of 24 hours. In the subsequent *chase*, cells are washed and ^{14}C valine is removed from the medium and replaced by a surplus of non-radioactive valine. Resultantly, the decrease in ^{14}C-labeled proteins and increase in free ^{14}C valine can be used as a readout of protein degradation. The ^{14}C valine liberated during the first hours of the chase is a result of degradation of short-lived proteins predominantly degraded by the proteasome (Henell et al. 1987). A chase period of 3 h is therefore included to allow degradation and wash-out of short-lived proteins, before the autophagy-inducing conditions are applied and the chase allowed to continue for 4 h. Whereafter the cells are harvested and separated into an acid-insoluble protein fraction and an

acid-soluble fraction representing the degraded proteins. By measuring the radioactivity in both fractions the ratio of free ^{14}C valine to total ^{14}C radioactivity allows for the calculation of degradation rate. Although long-lived proteins are prototypical bulk autophagic cargos, a drawback of the method is that it also measures non-lysosomal degradation. Even though the chase-step enrich the contribution from long-lived proteins, non-lysosomal degradation accounts for about 70-80% of the protein degradation taking place under nutrient-rich conditions in mammalian cell lines (Fuertes et al. 2003, Engedal et al. 2013). This gives the method a high background signal, but even so, the autophagic fraction can be identified by inclusion of an autophagic inhibitor, such as SAR405, or by silencing key autophagy components such as ULK1/ULK2 or ATG13, in a highly reproducible and sensitive manner due to the radioactive-based readout. Like the LDH sequestration assay, the LLPD assay have the benefit of being compatible with any mammalian cell line, also primary cell lines.

mKeima-based assays

The autophagy flux reporter mKeima was established by Katayama et al., 2011, as a sensitive and quantitative method to monitor the entire flux of autophagy, including lysosomal fusion. Monomeric Keima (mKeima) is a pH-responsive fluorescent protein resistant to lysosmal proteases. As such, it can provide a cumulative readout of autophagic activity as it stably accumulates in the lysosome and undergoes a change in chromophore resting charge state upon trafficking to lysosomes (pH~4.5) (Katayama et al. 2011). As a pH-responsive fluorescent protein, mKeima has an emission peak at 620 nm and a bimodal excitation spectrum peaking at 440 nm and 586 nm corresponding to the neutral and ionized states of the chromophores phenolic hydroxyl moiety (Violot et al. 2009). Since mKeima is resistant to lysosomal proteases, its accumulation in autolysosomes can be observed as punctate structures by fluorescence microscopy excited at 586 nm. Moreover, autophagic flux of mKeima can be measured in a highly quantitative manner by flow cytometry as the ratio of mKeima excited at 586 nm, primarily mKeima present in the autolysosomes (*acidic mKeima*), to mKeima excited at 440 nm, primarily mKeima present in the neutral environment of the cytosol or autophagosomes (*neutral mKeima*) (Figure 11). An advantage with the flow cytometry-based analysis of mKeima trafficking is that the analysis software allows you to derive the ratio per cell as a third analysis parameter. This eliminates the impact of different expression levels of individual cells. However, as mKeima has a broad bimodal excitation spectrum, the assay fits poorly with various live/dead stains without compensation. mKeima can also be detected by immunoblotting, however, this analysis method provides little information if mKeima is not

fused with a protein of the autophagic pathway. Sequestration of such a fusion protein into lysosomes gives the appearance of a “processed”/cleaved mKeima band by immunoblotting as the protein linked to mKeima is cleaved of and degraded, while mKeima itself is resistant to degradation. Although, mKeima is acid-stable, the appearance of a denaturing mKeima fragment can be seen by immunoblotting because of heat-induced hydrolysis of the N-acyl imine in mKeima (An et al. 2018)(Paper III). mKeima can be exploited as a cytosolic cargo or conjugated to cytosolic proteins to study bulk autophagic sequestration, or to track flux of autophagic components such as LC3 and p62. Moreover, mKeima can be conjugated to specific proteins or targeted to specific organelles to study selective autophgay, such as mitophagy, ER-phagy or ribo-phagy.

In contrast to the LDH sequestration assay and the LLPD assay, the mKeima-based assays do not rely on an experimental block in autophagy to accumulate a product (LDH sequestration) or calculate the autophagic degradation fraction (LLPD). The non-degradable nature of mKeima eliminates the need for such additional inhibitors to assess autophagic degradation. This is particularly advantageous with respect to the complete assessment of autophagy, as inhibiting lysosomal proteolysis precludes the evaluation of lysosomal function, and may affect the routing of cargo, such as NPs, to the lysosomes. However, a drawback compared to LDH sequestration and LLPD, is that the assay requires genetic introduction of mKeima and the further establishment of stable cell lines. Because mKeima is relatively small (25 kDa), it presumably interferes very little with the target protein/organelle function. However, the effects of mKeima overexpression in mammalian cells are not well known. Additionally, it is not well elucidated whether accumulation of mKeima in lysosomes may affect autophagic processes.

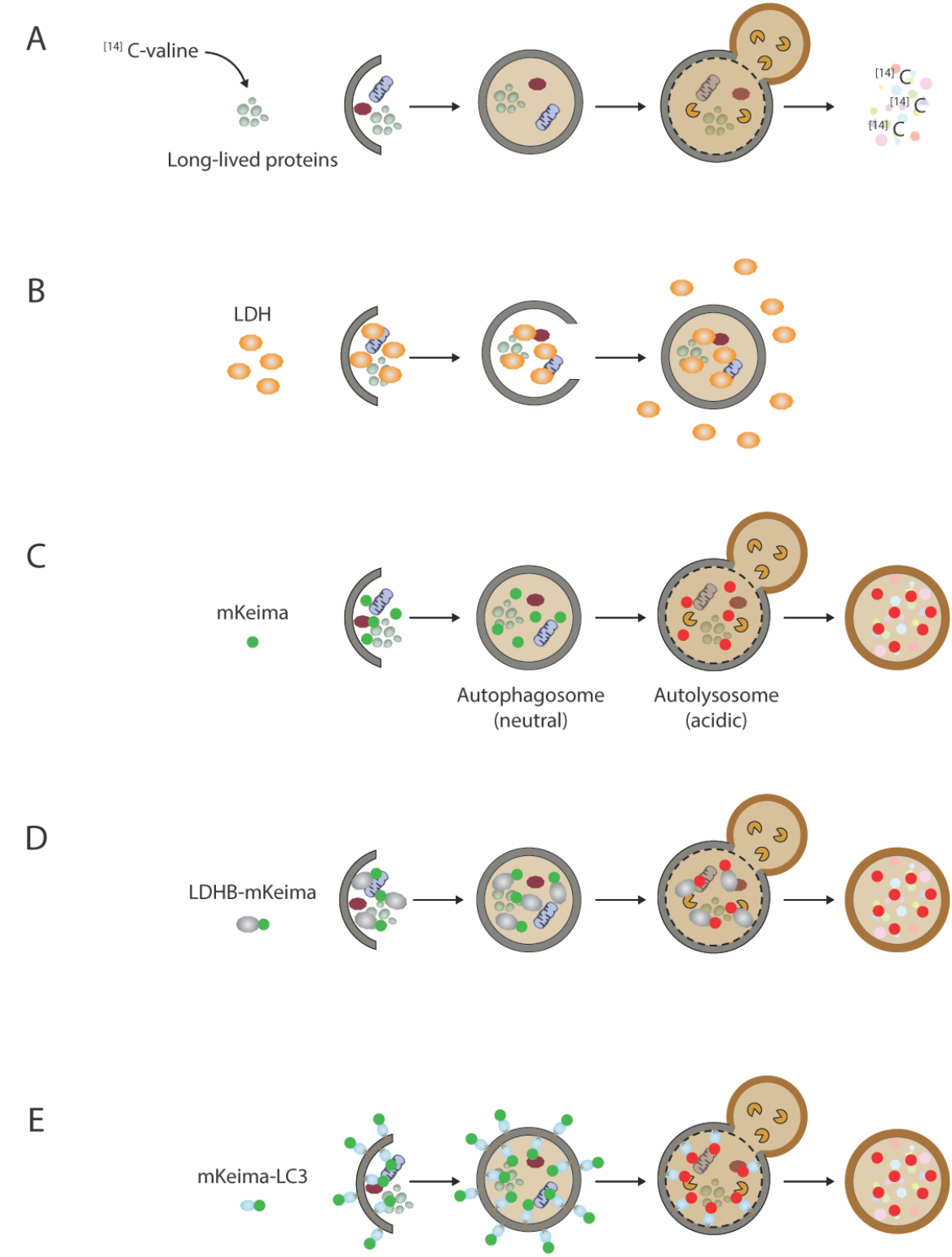

Figure 11. Schematic drawing of the various functional autophagy assays employed in this thesis. The long-lived protein degradation assay is a pulse-chase radiolabeling assay measuring the degradation of long-lived proteins (**A**). The LDH sequestration assays is a method to quantitatively measure the sequestration rate of bulk cytosolic cargo into closed autophagosomes, by using the cytosolic protein LDH as a marker (**B**). The autophagy flux reporter mKeima is a quantitative method to monitor the entire flux of autophagy, including lysosomal fusion. mKeima is pH-responsive and resistant to

lysosomal proteases, and as such provides a cumulative readout of autophagic activity as it stably accumulates in the lysosome and undergoes a change in chromophore resting charge state upon trafficking to lysosomes (pH~4.5) (**C**-**E**). In this work we thoroughly tested three variants of inducible mKeima-reporters; free cytosolic mKeima as a bulk autophagic cargo by itself (**C**), mKeima C-terminally tagged to LDHB, a cytosolic bulk autophagic cargo (**D**) and mKeima N-terminally tagged to LC3 to track lipidation of LC3 to the forming autophagosomal membrane (**E**).

In this work, we first tested cell lines stably expressing mKeima under the control of the CMV promoter. However, we quickly experienced that prolonged NP treatment up-regulated the expression level of the mKeima fusion-protein itself. We speculated whether it was caused by NP-induced activation of stress pathways upregulating the CMV promoter, as also described by others (Bruening et al. 1998, Wendland et al. 2015). Thus, in a collaboration with Dr. Lisa Frankel (Danish Cancer Society Research Center, Copenhagen, Denmark) we established Tet-inducible mKeima-reporters to assess the autophagic impact of NPs. In this work we thoroughly tested three variants of inducible mKeima-reporters, as illustrated in Figure 11. To monitor LC3 flux, mKeima was N-terminally tagged to LC3 to track lipidation of LC3 to the forming autophagosomal membrane (*mKeima-LC3*). To monitor bulk autophagic cargo degradation we established both mKeima C-terminally tagged to lactate dehydrogenase B (LDHB), as a cytosolic bulk autophagic cargo (*LDHB-mKeima*), and free cytosolic mKeima as a bulk autophagic cargo by itself (*free mKeima*). The inducible mKeima expression is also under the control of the CMV promoter, giving a relatively high expression level when first initiated. We titrated the doxycycline concentration and found 100 ng/ml doxycycline satisfactory for all assays, however with a varying induction time to give a valuable readout. mKeima-LC3, which is turned over at a high rate by autophagy, needed 24 hours to express a level of mKeima-LC3 which made it possible to assess both the accumulation of a cleaved mKeima band (25 kDa) and the disappearance/usage of the total mKeima-LC3 band (39 kDa) by immunoblotting. The LDHB-mKeima-reporter, as a bulk autophagic cargo, needed 48 hours of induction to give a high enough expression level of LDHB-mKeima to give a measurable band of cleaved mKeima after degradation. The free mKeima-reporter, which could not be assessed by immunoblotting, induced a measurable level of acidic mKeima by flow cytometry after 24 hours with doxycycline.

The inducible mKeima-system became advantageous for us in several ways. First of all, we induced expression of the mKeima-reporter for a given time, whereafter the doxycycline was washed away and the NP treatment added. This eliminates the possibility of the NP-induced

stress affecting the transcription level of the construct and obscuring the results. Furthermore, having a given amount of the mKeima-reporter, provided a reproducible and, by flow cytometry specifically, very sensitive readout of the degradation rate of that given amount by the various autophagy inducers (Torin1 and EBSS), as well as by the NP treatment. Measuring the cleaved level of mKeima by immunoblotting is naturally not equally sensitive and small changes in autophagic turnover rates are difficult to evaluate by this analysis method. In addition to providing a better measure of mKeima degradation by the treatments, inducing a given amount to a great extent diminish the impact of accumulated mKeima in the lysosomes prior to the conditions we were interested in assessing. We do not yet know the exact consequences of mKeima accumulating in the lysosomes, and how it may affect the autophagic process or even NP internalization. Hence, we kept the induction-time with doxycycline in our assays to a minimum, using 24 hours when adequate and 48 hours when required, keeping the mKeima accumulation low.

In Paper III we were interested in short-term effects of NPs on autophagic degradation capacity, without the interference from transcriptional upregulation. This is particularly important, as more than 20 transcription factors have been linked to long-term regulation of autophagy under various forms of stress (Füllgrabe et al. 2016). LDHB-mKeima holds the advantage over free mKeima, that it produces a cleavage product that can be assessed by immunoblotting, as a more visual readout in addition to the highly quantitative detection by flow cytometry. Notably, the mKeima-LC3 assay did pose some issues in interpretation of autophagic flux of our data, as PBCA treatment inhibited endogenous LC3 lipidation. Thus, when a certain treatment is found to inhibit mKeima-LC3 degradation, determining endogenous LC3 flux with a lysosomal inhibitor would indicate whether the blockade is at the lipidation step or at the final lysosomal degradation step.

Pharmacological inhibitors

Throughout this work we have used pharmacological inhibitors to assess the involvement of different stress-induced signaling pathways, to elucidate cell death mechanisms or to assess effects on the autophagic-lysosomal degradation pathway. Pharmacological inhibitors are useful tools to rapidly block the function of proteins, without depleting the protein in question (which is a more time-consuming process). Consequently, while the protein may lack a certain

activity it may still be able to interact with binding partners or assemble into macromolecular complexes. This can induce off-target effects and unforeseen feedback activation or inhibition. The efficacy of pharmacological inhibitors depends on specificity (its lack of binding to other proteins), potency (the concentration needed to inhibit the enzyme), membrane permeability (often requiring short preincubation for effect), solubility and chemical stability. To ensure few side effects and low toxicity, inhibitor concentrations were titrated, and we used as low concentrations as possible while still maintaining functional inhibition. Inhibitory effects were evaluated by specific downstream phosphorylation targets using immunoblotting for all inhibitors used, but also as functional increase or inhibition in autophagic flux by the various inhibitors of the autophagy pathway. Furthermore, all inhibitor studies in this work were corroborated by alternative methods such as genetic knock-down or knock-out of the protein, or by using additional inhibitors against the same enzyme or other members of the target signaling pathway.

siRNA-mediated depletion of proteins

Small interfering RNAs (siRNAs) are powerful tools to investigate protein function in signaling pathways. In this work, siRNA-mediated depletion was used to elucidate stress response pathways and how they mediate protective responses to the NP insult, such as transcriptional upregulation of certain genes. Additionally, siRNA-mediated depletion of proteins was used to corroborate results by pharmacological inhibitors (and vice versa) in the aim to rule out possible off-target effects. siRNAs ideally bind only the target protein's RNA/mRNA sequence and interfere with protein translation, thus reducing protein expression (short-term). Compared to the immediate effects of pharmacological inhibitors, siRNAs usually take days to have effect depending on the turnover rate of the target protein. As many enzymes act in concert to maintain cellular homeostasis, this may allow time for compensatory mechanisms obscuring the phenotypic readout. Moreover, the depletion of a protein by siRNA is not necessarily complete, and for many proteins a small amount can still be sufficient to bypass the potential phenotypic effect. Consequently, in our studies knock-down efficiency was always checked at protein level by immunoblotting. Non-specific binding to similar mRNA sequences can cause off-target effects, cellular toxicity and activate innate immune responses. To account for this, we; i) titrated siRNA concentrations to use as low concentrations as possible (to reduce possible off-

target effects and toxicity), ii) tested several siRNAs against the same target (to evaluate response specificity), and iii) used a scramble control from the same producer as our target (to evaluate/eliminate effects from induction of immune/interferon response).

Immunoblot analysis

Immunoblotting exploits the specificity inherent in antigen-antibody recognition to detect specific proteins or protein modifications in a sample of tissue homogenate or extract. Hence, immunoblotting lack the ability to distinguish cellular subpopulations, as it only allows for evaluation of the average expression of a protein in your sample. Although immunoblot is considered rather a qualitative method, today's increased sensitivity and stability of the detection signal has improved the accuracy of quantification. Quantitative analysis of immunoblotting depends on an established correlation between the detection signal and the amount of protein, and as such it is important not to overload the membrane with protein. Furthermore, to make comparisons between samples, all samples must be within linear range of the signal intensity (without saturated pixels) and acquired with the same settings and run on the same gel. In this work we have used immunoblotting for relative quantifications, and consequently, a well-known loading control protein that is unaffected by the experiment conducted must be added to make sure differences in signal intensity is not an effect of different protein loading. Negative and/or positive controls for your specific protein or protein modification is important to make sure that absence or presence of a band is due to absence or presence of that specific antigen, and not solely a problem with the antibody or any other step of the experiment. In our experience, if appropriate care is taken (from cell seeding through lysate preparation, gel application, blotting and immunostaining) highly reproducible and valuable immunoblotting data can be obtained.

Flow cytometry

Flow cytometry is a highly quantitative analysis technique that simultaneously measure and analyze multiple properties of each particle separated in a fluid stream. These characteristics include relative size, internal complexity (granularity) and fluorescence intensity. As the data

from each event are collected, flow cytometry provides information about subpopulations within the sample, as opposed to immunoblotting. Furthermore, the scattered and fluorescent light can be used to gate for (single out) these subpopulations, and hence exclusively analyze certain subpopulations. This was valuable to us as we were interested in assessing cellular effects of subtoxic PACA NP concentrations. By including a live/dead stain in our samples, such as propidium iodide or Hoechst, we could exclude dead or dying cells from our analysis of autophagic turnover or redox accumulation. However, the flow cytometry analysis, relies on the practitioner understanding how to draw up cell population gates accurately, as incorrectly placed gates may obscure the results. The mKeima fluorophore has a very broad bimodal excitation spectrum, incompatible with any of the live/dead stains available in our lab without compensating the spectra overlap. Since treatment with PACA NPs slightly changed the granularity of cells compared to control, we did not want to solely rely on light scattering for our gating strategy. Consequently, we decided to read each sample twice. First to collect the fluorescence emission from the mKeima fluorophore and secondly, after adding propidium iodide, to be used as a template for setting gates accurately. Importantly, the flow cytometry measurements of autophagic turnover nicely complemented the immunoblotting analyzes of the same assay.

DISCUSSION

The unique properties of NPs are exploited to develop drug delivery systems that maximize drug efficacy and minimize side effects for the patient. Due to their unique characteristics, NPs can increase solubility, circulation time and bioavailability of a drug, promoting a greater accumulation at the target site. Nonetheless, efficient drug delivery requires both internalization of the drug-loaded NP and release of the drug once internalized (Vauthier 2019). One key feature of NPs is their increased surface area to volume ratio, altering the material's mechanical, thermal and catalytic properties compared to known bulk properties. Upon interaction with living cells these altered properties may lead to undesired and unforeseen physiological consequences, contributing to the large gap between the development of novel medicines and clinical translation (Fu et al. 2014, Mohammadinejad et al. 2019). To understand the true potential and limitations of NPs as drug delivery systems, we need to decipher the consequences of their interactions with biological systems. In this work we have compared the cellular impact of three highly similar PACA NPs to increase our understanding of the dynamic and varied interactions that arise between NPs and the biological environment. We here demonstrate that even subtle differences in the NP structure significantly impact the NP-induced cellular stress responses. Both the type and degree of stress varied between the PACA variants, impacting the UPR, ISR and autophagy differently. This in turn led to differential transcriptional activation and possibility of resolving the stress by for instance increasing the antioxidant response or autophagic degradation. Consequently, our data illustrate how the specific characteristics of each PACA NP impacts the variation in cytotoxicity and mode of cell death.

Minor alterations in the nanoparticle polymer composition affect the nanoparticle-cell interaction and elicit particle-specific cellular stress

All the PACA variants, activated the ISR with the common effect of eIF2α phosphorylation. The POCA NP and high concentrations of the PEBCA NP induced ER stress. This was seen by the activation of PERK and its downstream effectors ATF4 and CHOP, and by accumulation of XBP1s, the downstream effector of IRE1 activation. Despite both NPs evoking ER stress, the NP-induced level of cytotoxicity was different. The PBCA NP only marginally induced

PERK activation, but instead induced oxidative stress seen by increased intracellular ROS and depletion of the cellular GSH reserves. PBCA was further found to preferentially activate the HRI kinase of the ISR, likely downstream of the induced oxidative stress as co-treatment with NAC or GSH abolished ISR activation. Treatment with PEBCA was also found to accumulate ROS, however having little effect on cytotoxicity before the cells were further challenged by cystine starvation. This emphasizes how NP-induced sub-toxic cellular stress can compromise cell function and render the cells more vulnerable upon additional insults. Treatment with the PEBCA NP was found to elevate LC3-II protein levels and upregulate *LC3B* mRNA expression via the ISR and ATF4, in manner that did not alter LC3 flux or autophagic cargo degradation. PBCA also upregulated *LC3B* mRNA levels via the ISR and ATF4, but surprisingly caused a redox imbalance-dependent inhibition in LC3 lipidation. The PBCA induced redox imbalance was further demonstrated to induce autophagic cargo degradation at moderate levels and inhibit autophagic cargo degradation at high levels. Treatment with the POCA NP strongly accumulated LC3-II protein levels without an effect on *LC3B* mRNA levels. The accumulation was found to be associated with reduced degradation of LC3-II as autophagic cargo degradation was inhibited by POCA. The outcome of the NP-cell interaction as reflected by cell viability of each PACA variant, seems to be determined by the balance between the pro-survival or pro-death responses induced.

The PACA nanoparticle variants are internalized to the same degree

NPs elicit a wide range of cellular responses depending on their physicochemical properties, which in turn determine their intracellular concentration, duration of contact, subcellular distribution, and interactions with biological molecules (Nel et al. 2009). However, the PACA variants investigated in this thesis exhibit very similar physicochemical characteristics with a size range from 133-153 nm, polydispersity between 0.13-0.16 and surface charge of -2.4 to -3.4 mV (Paper I), not obviously promoting such different responses. Cellular uptake of NPs is a premise for their application in drug delivery and a major determinant of NP induced stress. Size, shape, surface charge, surface hydrophobicity/hydrophilicity and surface functionalization directly affect the NP uptake level, endocytic route, and cytotoxicity (Foroozandeh et al. 2018). Moreover, dispersion, aggregation and agglomeration state has also been demonstrated to be important for NP internalization and cellular responses (Liao et al. 2011, Wang et al. 2011, Huang et al. 2015). The toxicity of graphene and graphene oxide NPs were found to depend on their state of aggregation, where aggregated NPs displayed a higher

toxicity to adherent cells possibly due to faster sedimentation on top of the cells. However, fully dispersed sonicated NPs, yielding more cell-contactable reactive oxygen groups on the surface, displayed a higher hemolytic activity in suspended red blood cells (Liao et al. 2011). Huang et al., 2015, found that iron oxide, gold and silica NPs induced autophagy in a dispersity-dependent manner. Aggregated NPs induced significant autophagic effects compared to well-dispersed NPs, and they speculate whether increased uptake of NP aggregates is stimulating autophagy (Huang et al. 2015). The state of dispersion is affected by the way in which we administer particles to cell culture, which have been shown to significantly impact the particle cell-interaction (Moore et al. 2019). As such, the three PACA variants were handled equally in our experiments and all experiments were performed on fully dispersed NPs. The cellular uptake of the PACA NPs in this work was found to be energy dependent in a panel of cell lines, thus requiring an active uptake mechanism, and the PACA variants were internalized to the same degree after 2 hours of incubation (Paper I). However, the cell-associated POCA fluorescence was slightly higher than the PEBCA- and PBCA-fluorescence both during active uptake at 37 °C and when active uptake is inhibited at 4 °C. The PACA polymers have alkyl side chains of increasing hydrophobicity, where POCA is the most hydrophobic and PBCA is the least hydrophobic. Hence, the POCA NP may associate better with the cell membrane given the more hydrophobic monomer. This may affect the kinetics of POCA uptake at 37 °C and explain the slight degree of cell adhesion at 4 °C. In corroboration of this Sulheim et al, 2016, found that PC3 cells internalize POCA NPs to a larger extent than PBCA NPs after 3 hours. However, the uptake kinetics was found to be highly cell-type dependent as RBE4 cells internalized PBCA NPs to a higher degree than POCA NPs after 24 hours while the initial uptake efficiency was approximately equal (Sulheim et al. 2016). However, it cannot be excluded that there may be cell-dependent differences also in the exocytosis of particles, an effect that is revealed after prolonged uptake. In their studies the uptake of POCA NPs in PC3 cells was eightfold higher than PBCA after 3 hours (Sulheim et al. 2016), in vast contrast to our studies with only a slight difference in cell-associated fluorescence independent of cell-type (Paper I). Moreover, across the panel of cell lines used in Paper I, there was no correlation between the magnitude of POCA cell association and cellular toxicity. Even so, we cannot exclude the possibility that increased cell contact of the POCA NP compared to the PEBCA and PBCA NP, influence the differences in impact and type of cellular responses induced by the PACA variants.

Degradation of PACA nanoparticles gives alkyl alcohols of varying hydrophobicity

Given that the PACA NPs are internalized to the same degree, differences in intracellular concentrations are not likely a major cause of the variable cellular stress responses and cytotoxicity. However, the particle-specific end-products of degradation, once inside the cell, may contribute to such variations although the degradation of PACA NPs have been demonstrated to be slow *in vitro* (Sulheim et al. 2016). The main mechanism of PACA NP degradation is described to be hydrolysis of the ester bond of the alkylcyanoacrylate releasing a water soluble alkyl alcohol and cyanoacrylic acid (Vezin et al. 1980, Vauthier et al. 2003). Short-chain aliphatic alcohols have a high affinity for cellular membranes (Hunt 1975), and their insertion into cellular membranes may disrupt the structural organization of the lipid bilayer and membrane integrity (Lyon et al. 1981). Furthermore, the hydrophobicity of the alcohol has been correlated with its potency to alter membrane integrity and cause LDH release (McKarns et al. 1997). As such, the hydrophobicity of the alkyl alcohols released upon PACA polymer degradation, ethyl-butyl alcohol, butanol, or octanol, determines how readily it elicit possible toxic effects through hydrophobic interactions with cellular membranes. As a result, POCA degradation giving octanol, the most hydrophobic alkyl alcohol, as an end-product will likely affect membrane properties to a greater extent than PEBCA and PBCA. In support of this, octanol induced calcium release and apoptosis in neural crests at micromolar concentrations, whereas millimolar concentrations of butanol were required (Garic-Stankovic et al. 2006). In comparison, the POCA NP readily induce apoptosis even at comparably low concentrations, whereas the PEBCA and PBCA NP does not.

PACA nanoparticle specific formation of protein corona may affect nanoparticle-cell interactions

From our investigations, it seems likely that the PACA variants induce particle-specific effects depending on the NP-cell interaction, and not variations in cellular uptake. Investigations of the particle specific formation of protein corona have not been in the scope of this work. However, it is likely that the exact composition of the protein corona surrounding the PACA variants mediates the variability in cellular responses.

The most used method to reduce protein adsorption is to coat the NP with PEG. The PACA NPs were made by mini-emulsion polymerization, a one-step synthesis method incorporating PEG groups as the anionic polymerization of the PACA monomers is initiated

(Mørch et al. 2015). This method of preparing PEG-coated polymeric NPs results in chemical bonding of PEG to the PACA polymer chains, and hence assures tightly bound PEG at the particle surface (Peracchia et al. 1997). However, it does not assure complete surface coverage of PEG, as the PEG groups might be sterically hindered by the stabilizer (miglyol) in the polymerization procedure (Mørch et al. 2015). It has been demonstrated that the method of fucoidan-coated NP synthesis determines the polysaccharide chain conformation on the nanoparticle surface, and that this has a large impact on the cytotoxicity induced in J774 macrophages (Lira et al. 2011). Hence, it may be possible that the specific PACA polymer chain used for synthesis gives a slight difference in the spatial arrangement of the PEG-chains, and in this way also affect the accessibility of the NP core to the surrounding medium. Consequently, although the three PACA variants are of similar size, shape, and surface charge, the specific PACA polymer having varying degree of hydrophobicity may cause differences in the protein corona, governing the biological identity of the NP (Cedervall et al. 2007). Chandran et al., 2017, showed in a study comparing different surface functionalization of gold NPs, that surface chemistry is more important for the protein corona composition than size of the NPs (Chandran et al. 2017). Furthermore, both hydrophobicity and the chemical motif of the head group, being branched, cyclic or linear, are found to control the nature and chemical identity of the protein corona under physiological serum conditions (10%) (Saha et al. 2016). For instance, the number of apolipoproteins and immunoglobulins was found to decrease with increasing NP surface hydrophobicity, and NPs with branched or cyclic head groups generally adsorbed less proteins than linear structures with similar hydrophobicity (Saha et al. 2016). This was corroborated by a study investigating the impact of the hydrophilicity of the polymer layer on protein adsorption. Although the total amount of adhered protein was equal in human blood plasma, the protein pattern was different determining the NP-cell interaction (Simon et al. 2018).

The nature of the NP surface facilitates the interaction with the cell membrane. Anionic gold NPs have been demonstrated to indirectly disrupt ion channel function by altering the mechanical properties of the surrounding lipid bilayer, a proposed important mechanism by which NPs induce biological effects (Foreman-Ortiz et al. 2020). Protein-NP interactions has been proposed as the major contributor to reduced ion transport in the presence of cationic ZnO NPs (Bryant et al. 2017) and cationic polymer NPs (Fologea et al. 2013). Such interference with transmembrane transport mechanisms have been demonstrated for several NPs affecting intracellular ion homeostasis and signaling pathways, and ultimately nanotoxicity (Yin et al.

2019). The NP-cell interaction may lead to alterations in membrane fluidity, the composition of micro-domains, or membrane curvature (Nel et al. 2009). The dynamic interaction of such microdomains with the underlying cytoskeleton may regulate cell function and adaptation to changing environments (Head et al. 2014). Graphene and fullerene NPs can be adsorbed onto the lipid membrane and insert in its hydrophobic regions and deplete the surrounding bilayer for cholesterol, having consequences for the functionality of the lipid membrane (Sastre et al. 2017, Puigpelat et al. 2019, Santiago et al. 2019). Such membrane alterations are known to affect the activity of membrane proteins like receptors, enzymes, ion channels, and nutrient transporters, and have been proposed to signal membrane stress to the cell interior (Mouritsen 2011).

The specifically acquired protein corona defines the NPs biological identity, and hence, what is seen by the functional machinery of cells, affecting cell internalization, biodistribution and cytotoxicity (Owens et al. 2006, Lundqvist et al. 2008, Aggarwal et al. 2009). Certain protein components play key roles in cellular uptake due to receptor-ligand interactions (Binnemars-Postma et al. 2016). For instance, presence of apolipoproteins is described to be significant for cell association (Palchetti et al. 2016), whereas glycans are shown to negatively impact cell adhesion (Wan et al. 2015). The binding of fibrinogen to the surface of silver and gold NPs have been shown to provoke an inflammation response as this cause fibrinogen to unfold and activate the Mac-1 integrin receptor (Deng et al. 2011, Kharazian et al. 2018). Furthermore, in the presence of a protein corona, black phosphorous nanomaterials were found to cause immune perturbations (Mo et al. 2018) and silver NPs to significantly increase ER stress (Persaud et al. 2019). Protein adsorption onto cadmium sulfide NPs enhance the expression of the cell surface receptor FcγRIIB, triggering the AKT/Caspase 3 pathway and cell apoptosis (Wu et al. 2018). It is increasingly accepted that the composition profile, formation, and protein conformational changes of the protein corona may affect NP-cell interaction, and that the nature and extent of these interactions participate in determining the biocompatibility of a given NP (Liu et al. 2020). Thus, identity-differences in the protein corona of the PACA variants may affect the nature of the NP-cell interaction and the induction of cellular stress.

The PACA-induced type and magnitude of cellular stress affect nanoparticle cytotoxicity

Regulation of protein synthesis holds the advantage of enabling an immediate and reversible response upon cellular stress (Holcik et al. 2005). Global translation is reduced in response to almost all types of cellular stress, saving energy, preventing synthesis of unwanted proteins, and reducing the ER workload. However, remarkably, the stress-induced attenuation of global translation is accompanied by selective translation of proteins involved in resolving the stress.

POCA cause ER stress induced cytotoxicity, whereas PBCA and to a minor degree PEBCA cause oxidative stress-induced cytotoxicity

The accumulation of ubiquitinated proteins and induction of heat shock proteins is a clear sign that ER homeostasis is challenged by the PACA NPs (Paper I). Activation of the UPR/ISR is aimed at reducing the ER workload and increasing transcription of ER chaperones (Hetz et al. 2020) to allow the cell to reestablish full ER function. All the PACA variants, activated the ISR with the common effect of eIF2α phosphorylation and attenuation of global protein translation. The POCA NP, specifically, and high concentrations of the PEBCA NP was found to activate the UPR/ISR downstream of ER stress as observed by PERK activation and accumulation of XBP1s downstream of IRE1 activation. Such an ER stress-specific response was only marginally observed by PBCA. Instead, PBCA was found to preferentially activate the HRI kinase of the ISR, which have been described to be activated downstream of heme deficiency (Rafie-Kolpin et al. 2000) and oxidative stress (Lu et al. 2001). Both PEBCA and PBCA was found to induce oxidative stress, wherein PBCA was also demonstrated to reduce the cellular GSH reserves. The PBCA-induced oxidative stress was found to activate the ISR, as cotreatment with NAC or GSH abolished ISR activation.

POCA exerted the greatest cytotoxicity in the cell lines that accumulated ATF4, CHOP and XBP1s to the largest extent. This likely indicates that the magnitude of ER stress induced, correlates with the level of cytotoxicity. Several NPs are demonstrated to cause cytotoxicity associated with ER stress (Cao et al. 2017) however the underlying mechanism is largely unknown. CdTe quantum dots have been described to localize to ER and alter ER morphology causing dilatation of the ER cisternae (Yan et al. 2016). Gold NPs have also been described to localize to ER and induce accumulation of misfolded or unfolded proteins by impairing the

ERAD activity (a key degradation process for misfolded proteins) (Guofang et al. 2021). Zinc oxide NPs, and its related zinc ions, were found to induce ER stress due to its induction of oxidative stress as cotreatment with NAC abolished the ER stress markers induced (Chen et al. 2014). A study investigating the effect of silver NP size and protein corona, found that the presence of a protein corona significantly increased ER stress even though the protein corona reduced AgNP internalization (Persaud et al. 2019). This further supports our main hypothesis that the acquired protein corona may dictate the type of cellular stress induced. In contrast to the POCA NP, alterations in cellular redox capacity seems to be a key mechanism for both PEBCA and PBCA induced cytotoxicity, as has been described for many NPs (Wang et al. 2019). However, the PEBCA-induced ROS was found to have little effect on cytotoxicity before the cells were further challenged by cystine starvation (Paper I). Attenuation of global protein synthesis may increase the availability of amino acids important for producing antioxidants such as GSH, and as such alleviate the particle-induced oxidative stress temporary to initiate long term survival responses.

ER stress, ROS production and redox control have been described to be tightly connected (Zeeshan et al. 2016, Kritsiligkou et al. 2018). However, although we observe a prominent PERK activation by POCA indicative of ER stress, POCA does not lead to an immediate accumulation of ROS equal to PEBCA or PBCA. Furthermore, cotreatment with NAC or GSH does not rescue the POCA-induced cytotoxicity (Paper I). This indicates that the particle-specific stress is mediated by separate mechanisms. With regards to the relatively high cytotoxicity of POCA compared to the same concentration of PEBCA and PBCA, it seems that the POCA-insult leaves the cells with little/no ability to initiate pro-survival responses while the PEBCA- and PBCA-insult may engage such responses.

Pro-survival transcriptional responses induced by PEBCA and to a lesser extent PBCA protect against cytotoxicity

Regulation of translation provides the cell with the plasticity needed to respond rapidly, while regulation at the transcription level fine-tunes the long-term cellular response required to coordinate expression of stress-responsive genes affecting cell survival. ATF4 is a key stress-induced transcription factor downstream of p-eIF2α, activating expression of genes involved in redox homeostasis, amino acid metabolism, protein synthesis, apoptosis, and autophagy (Hetz et al. 2020). ATF4 also participates in a feedback loop dephosphorylating eIF2α to restore

protein synthesis (Kojima et al. 2003). All the PACA variants were found to accumulate ATF4, which for PEBCA and PBCA were confirmed to be accompanied by a significant increase in *ATF4* mRNA. We further demonstrated that ATF4 was accumulated downstream of PBCA-induced oxidative stress and p-eIF2α, and protected against PBCA induced cytotoxicity (Paper I).

Another important transcription factor in the antioxidant response is Nrf2. Our data from Paper I point to a more potent and prolonged Nrf2-mediated antioxidant response induced by PEBCA as the cause of the lower cytotoxicity from this particle. Nrf2 is kept at low levels under homeostatic conditions by Keap1-mediated ubiquitination and proteasomal degradation (Cullinan et al. 2004). However, under stress and exposure to ROS, Keap1 is inactivated resulting in Nrf2 stabilization and translocation to the nucleus to activate transcription of cytoprotective genes (Itoh et al. 1999). Notably, Nrf2 has been demonstrated to induce *ATF4* transcription under oxidative stress (Afonyushkin et al. 2010, Miyamoto et al. 2011). Furthermore, PERK activation has been described to directly phosphorylate Nrf2, causing Keap1-Nrf2 dissociation and Nrf2 activation (Sarcinelli et al. 2020). As high concentrations of PEBCA were demonstrated to activate PERK, one can speculate whether such a mechanism is contributing to the prolonged Nrf2 activation seen for PEBCA compared to PBCA. Moreover, PEBCA was demonstrated to induce ATF4 transcription in an Nrf2-dependent manner (Paper I) reinforcing the cytoprotective effects.

ATF4 and Nrf2-upregulation by PEBCA and PBCA were found to act as pro-survival pathways protecting the cells from ferroptosis. However, depletion of Nrf2 sensitized MDA-MB-231 cells to PEBCA to a higher degree relative to PBCA, indicating a more potent Nrf2-protection induced by PEBCA. Both PEBCA and PBCA induced transcription of the cystine transporter, *SLC7A11*, downstream of ATF4 and Nrf2. Depletion of SLC7A11 induced comparable cell death as double knockdown of ATF4 and Nrf2, underscoring the importance of upregulated cystine import in the inhibition of PEBCA- and PBCA-induced cytotoxicity. Cystine is rapidly reduced to cysteine once inside the cell. Intracellular cysteine levels are particularly important as the rate-limiting precursor in glutathione synthesis, which is critical in the cellular defense against oxidative stress (Griffith 1999). The pro-survival upregulation of SLC7A11 is associated with cytotoxic drug resistance by increased cellular detoxification (Okuno et al. 2003, Huang et al. 2005). Conversely, depletion of intracellular glutathione is associated with inhibition of cell growth (Schnelldorfer et al. 2000) and reduced drug resistance (Vanhoefer et al. 1996). In light of this, inhibiting the cystine transporter represent a potential

target for therapy of cancers that are critically dependent on uptake of amino acids for growth and viability (Gout et al. 1997, Timmerman et al. 2013), and an alternative method of depleting glutathione to overcome drug resistance (Gout et al. 2003). Corroborating this, both PEBCA and PBCA cytotoxicity was inversely correlated with cystine availability in MDA-MB-231 cells.

PEBCA may represent a NP that have the potential to increase drug resistance via the strong activation of pro-survival Nrf2 and SLC7A11. However, additional cystine starvation or depletion of the cystine transporter SLC7A11 made even the highly tolerable PEBCA particle potently cytotoxic, repressing the originally induced pro-survival pathways. As such an additional insult was needed to reveal the sub-toxic oxidative effects of PEBCA. Without the proper redox buffer capacity, the pro-survival responses induced by PEBCA were not able to maintain balance. In a treatment setting, there may be two ways of looking at this effect. It can compromise treatment outcome by the strong initiation of pro-survival responses, or it can be exploited by the therapeutic agent, once released, adding the additional insult needed for the NP to be highly cytotoxic, and in such a manner limit side effects to a larger degree.

PBCA rather represent a NP with the potential to contribute to reduced drug resistance, however with the consequence of potentially higher side-effects. From our observations it seems unlikely that inhibition of cystine import is the sole cause of PBCA induced oxidative stress and cytotoxicity, as cystine starvation was less cytotoxic than PBCA treatment. This indicates that an additional insult, such as GSH depletion, is caused by PBCA treatment which could be beneficial in overcoming drug resistance. For instance, an effect of GSH depletion concomitant to reduced cystine import, has been shown to be required for ferroptosis induction on HepG2 cells (Yu et al. 2016).

The PACA-induced type and magnitude of cellular stress affect the lysosomal intracellular degradation pathway, autophagy

In Paper II we investigated how the highly similar PACA NPs affect autophagy as an important cytoprotective pathway, and found that the PACA variants differentially affected the autophagosome marker LC3 and the autophagy pathway. PEBCA induced accumulation of

LC3-II as a transcriptional response without affecting LC3 flux or autophagic cargo degradation. Treatment with the POCA NP also led to a strong accumulation of LC3-II, but due to an inhibition in LC3 flux and autophagic cargo degradation. By contrast, PBCA did not affect LC3-II protein levels despite a transcriptional response and rather seemed to inhibit LC3-lipidation and autophagic cargo degradation.

Cellular uptake of NPs is strongly associated with various effects on autophagy, as most endocytic routes converge upon the lysosome (Stern et al. 2012). However, as discussed earlier our data indicate that the PACA variants are internalized to the same degree, giving a similar load of NPs in the endolysosomal system. In support of this our data did not demonstrate any differential induction in lysosomal dysfunction between the PACA variants. In fact, the PACA NPs did not seem to affect lysosomal homeostasis, as there was no significant change in the activity of the lysosomal enzyme cathespin D, and no galectin-3 puncta appeared upon PACA NP treatment, indicative of lysosomal permeabilization (Paper II). From our data, it rather seems that the differential effect on autophagy is caused by the biological identity of each PACA NP dictating its cellular interaction, inducing a variation in cellular stress responses. NP-induced cellular stress may affect autophagy in several ways as seen throughout Paper II and III of this thesis. At the transcriptional level NP may increase expression of important ATGs, as demonstrated for PEBCA and PBCA in Paper II, as an attempt to maintain autophagy or increase the degradation capacity. NPs may also interfere more directly with the autophagy process by acting on upstream signaling events, such as mTORC1 as addressed in Paper II and III, or PI3KC3 as shown for PBCA in Paper III. NPs may interfere with components of the autophagy machinery, such as LC3 with varying effects on autophagic turnover as demonstrated in Paper II and III, or inhibit the fusion of autophagosomes with lysosomes as shown for POCA in Paper II.

Transcriptional regulation of autophagy

Regulation of autophagy at the transcriptional level was first observed in yeast, where induction of autophagy by nitrogen starvation was found to upregulate Apg8p (Kirisako et al. 1999). Since then, several transcriptional regulators of autophagy have been identified. A key regulator, emphasizing the importance of the lysosome in the autophagy pathway is transcription factor EB (TFEB), the master regulator of lysosomal pathways (Sardiello et al. 2009, Settembre et al. 2011, Martina et al. 2017). NP-induced TFEB activation to increase

cytoprotective autophagy has been shown in cells treated with both silver (Lin et al. 2018) and polystyrene NPs (Song et al. 2015). Interestingly, however, TFEB or the related TFE3 (Martina et al. 2014) was not found to be involved in the PEBCA-induced increase of *LC3B* mRNA, although the highest concentration (100 µg/ml) led to a partial nuclear translocation of TFEB. One can only speculate whether the lack of effect on mTORC1 deactivation or lysosomal dysfunction by PEBCA treatment is keeping TFEB phosphorylated (Peña-Llopis et al. 2011, Settembre et al. 2011).

On the other hand, *LC3B* upregulation was found to be dependent on PEBCA and PBCA induced ISR activation. The transcription factor ATF4 has been shown to upregulate ATGs, including *LC3B*, under various stress (Rouschop et al. 2010, Rzymski et al. 2010, B'Chir et al. 2013, Luhr et al. 2019) and to enhance autophagosome formation upon treatment with silica NPs (Wang et al. 2018). Both PEBCA- and PBCA-induced *LC3B* mRNA increase was dependent on ATF4 downstream of the ISR. However, as POCA also induced a potent accumulation of ATF4 without an increased *LC3B* mRNA, accumulation of ATF4 alone does not seem sufficient to induce *LC3B* transcription. In Paper I, PBCA was found to induce ATF4 accumulation downstream of oxidative stress, and as such also *LC3B* mRNA levels was found to depend on PBCA-induced oxidative stress (Paper II). In contrast, POCA induced ATF4 accumulation via ER stress without a significant increase in ROS (Paper I). In that regard, it is tempting to speculate whether the rapid *LC3B* increase seen for both PEBCA and PBCA, requires additional input from oxidative stress-induced transcription factors such as FoxO1 (Kode et al. 2012) or Nrf2 (Mimura et al. 2019). However, where PEBCA manages to maintain autophagic degradation, the redox imbalance induced by PBCA eventually inhibits autophagic degradation through an additional oxidative stress related LC3 lipidation inhibition mechanism (Paper II and III). Although, ER stress has been proposed to induce cytoprotective autophagy by increasing expression of *ATGs* via ATF4 and CHOP (Rouschop et al. 2010, B'Chir et al. 2013), we do not observe this downstream of POCA-induced ER stress. Conversely, treatment with the POCA NP was found to inhibit autophagic degradation at a step prior to lysosomal fusion. However, as these studies did not demonstrate that the increased *ATG* expression leads to increased autophagic activity and is not merely a response to inhibited degradation it is hard to draw any conclusions. However, Engedal et al., 2013, showed that the ER stress inducers thapsigargin and A23187, despite activating the UPR and increasing *LC3B* mRNA, blocked autophagy at a step prior to phagophore closure. They further demonstrate that local calcium changes were sufficient to inhibit autophagy (Engedal et al. 2013).

Considering the variation in PACA-induced cytotoxicity and activation of pro-survival responses explored in Paper I, it is tempting to speculate that the stronger pro-survival response induced by PEBCA upregulates autophagy biogenesis components which maintains autophagic degradation throughout the stress. Whereas for PBCA, the GSH depletion and ROS accumulation as observed both in Paper I and III, inhibits LC3 lipidation despite transcriptional upregulation when the oxidative stress is too severe and increase cytoprotective autophagic degradation through MAPK activation when the oxidative stress is moderate. The severity of the POCA NP-insult blocks autophagic degradation, contributing to the high cytotoxicity of this particle.

Redox regulation of autophagy

Interestingly, complementation of LC3 flux studies with various cargo sequestration and degradation assays in Paper III revealed yet another NP-induced 'autophagy phenotype'. When assessing stimulated LC3 flux in RPE-1 cells, we found that PBCA at concentrations significantly inhibiting LC3 lipidation (12.5 µg/ml) had no effect on autophagic LLPD, and conversely increased autophagic LDH sequestration and cargo degradation as assessed by LDHB-mKeima and 'free mKeima'. The formation of LC3-II has been recognized as a hallmark of autophagy initiation (Kabeya et al. 2000). However, in contrast to yeast, mammals have several ATG8 orthologues, separated into two subfamilies based on sequence similarity; the GABARAPs (GABARAP, GABARAPL1 and GABARAPL2) and LC3s (LC3A, LC3B and LC3C) (Xin et al. 2001, He et al. 2003). Evidence is accumulating that LC3s are redundant for bulk autophagic degradation (Szalai et al. 2015, Engedal et al. 2016, Nguyen et al. 2016, An et al. 2018, Vaites et al. 2018) and that LC3 has functions that are not autophagy related (de Haan et al. 2010, Al-Younes et al. 2011, Monastyrska et al. 2013). Hence, cytosolic sequestration and cargo degradation can occur by way of other ATG8 orthologues even though PBCA is inhibiting LC3 lipidation. Conversely, the GABARAP subfamily have been described to be required for bulk autophagic sequestration and cargo degradation (Szalai et al. 2015, Nguyen et al. 2016). In line with this, we further demonstrated that PBCA treatment also inhibit GABARAP lipidation in RPE-1 cells. However, importantly, we do not see complete inhibition of LC3 or GABARAP lipidation by treatment with 12.5 µg/ml PBCA, the concentration where autophagy is not reduced. PBCA lowers LC3-II levels to about 30% of that induced by Torin1, indicating that the remaining ATG8s can be sufficient to fully support autophagy. Furthermore, our data implies that PBCA at 6.25 µg/ml activates an LC3 flux-independent form of autophagy

that is still dependent on the canonical autophagy machinery components, e.g., Vps34, ULK1/2, and ATG13 (Paper III). Further studies would be needed to identify the key players in this scenario.

Intriguingly, both the inhibition in LC3 lipidation and the autophagy potentiation through MAPK activation was found to be downstream of PBCA-induced oxidative stress. ROS serve as important signaling molecules to maintain homeostasis when handled properly, however, when improperly regulated, excessive ROS mediates oxidative stress and cellular damage (Ray et al. 2012). In this regard, ROS can activate autophagy as a cytoprotective response to possibly eliminate the sources of ROS, such as damaged mitochondria (Wible et al. 2018). Some even propose ROS to be essential in activation of stress-induced autophagy (Scherz-Shouval et al. 2007, Chen et al. 2009, Li et al. 2013, Qiao et al. 2015). In line with this, our data from the PBCA NP suggest that there is a turning point in the NP-insult where the ROS is no longer manageable, and the oxidative stress become excessive. The lower concentrations of PBCA initiate cytoprotective responses, amongst which the autophagic degradation capacity is increased most likely by MAPK activation acting on the PI3KC3 complex. Conversely at higher PBCA concentrations, the cells are no longer able to regulate the ROS accumulation and excessive ROS then mediates cytotoxic effects, LC3 lipidation-inhibition and in the end inhibition of autophagic cargo degradation.

The turning point concentration of 12.5 µg/ml PBCA, in some way captures the cells at a stage where they still fight for survival aiming to clear dysfunctional components and reduce the oxidative stress, as the oxidative reactions are gaining ground exhausting the available antioxidants and accumulating ROS. To the best of our knowledge, PBCA is the first NP shown to reduce LC3-II protein levels due to an inhibition in lipidation. The rapid loss in LC3-II suggest post-translational modifications, rather than transcriptional regulation of LC3 levels as was also verified in Paper II. The inhibitory phenotype was mimicked by H_2O_2, and prevented by co-treatment with excess of antioxidants, strongly indicating that the inhibition is downstream of redox imbalance. Frudd et al., 2018, recently described a mechanism whereby direct oxidation of ATG3 and ATG7 impaired autophagy (Frudd et al. 2018). When autophagy is active, the stable thioester bond with LC3 is lost, and the authors suggest this exposes the thiol groups of ATG3 and ATG7 for oxidation. Moreover, in a cell-free system, they demonstrated that increased levels of GSSG (oxidized GSH) inhibited the enzymatic activity of the catalytic thiols on ATG3 and ATG7 by S-glutathiolation (Frudd et al. 2018). The

implication of altered GSH levels in their molecular mechanism of LC3 lipidation inhibition, supports the possible involvement of this mechanism in PBCA-induced lipidation inhibition.

Fine-tuning the autophagic response with the level of NP-induced redox imbalance is an intriguing finding. Especially, as oxidative stress is an underlying mechanism for NP-induced cytotoxicity by various NPs (Nel et al. 2006, Wang et al. 2019). It further -refers to the discussion of the cystine transporter and ferroptosis being targets of therapy as drug-resistant mesenchymal cancer cells are sensitive to GSH depletion (Gout et al. 2003, Lu et al. 2018, Wu et al. 2020). From both Paper I and III, we know that PBCA lead to a reduction in GSH as well as an accumulation of ROS and an increase in lipid peroxidation. Furthermore, that this induce ferroptosis by PBCA treatment and that the cytoprotective responses are highly concentration-dependent, increasing antioxidants and autophagy at lower concentrations. If the aim of the treatment is killing cancer cells, a too low concentration of PBCA could compromise the treatment outcome as cytoprotective autophagy is initiated, as is proposed for chitosan NPs combined with doxorubicin (Wang et al. 2018). Conversely, for neurodegenerative disorders, enhanced autophagy is described to decrease pathogenesis and such an effect would benefit the intended treatment (Towers et al. 2016).

Functional assays to study nanoparticle effects on autophagy

As the 'nanoparticle and autophagy'-field progress, it should be with the aim to implement functional assays to a high degree. To be able to distinguish the end-result of the specific PACA NPs effect on autophagy in this work, functional assays have been invaluable. As previously addressed, we have here demonstrated various ways of how LC3-II, as a marker of autophagosomes, can be regulated without a common effect on autophagic degradation activity. Several papers investigating the autophagic response to NP treatment assess flux of common markers such as LC3 and p62 (Manshian et al. 2014, Wu et al. 2014) or look at transcriptional regulation (Lopes et al. 2016, Popp et al. 2018). However, few papers relate these effects to functional autophagic turnover, which also complicates the comparison of various NPs. Therefore, a large part of this work was to establish and compare methods to functionally evaluate the autophagic outcome of NP-cell interaction.

The initial investigations of autophagy used transmission electron microscopy (TEM) to assess formation of autophagosomes and cargo sequestration (Moe et al. 1962, Hruban et al. 1963). However, TEM is not a quantitative method and technical challenges regarding stage of

maturation and closure of the vesicles prompted complementary assays. The LDH sequestration assay was initially developed to assess macroautophagic cargo degradation in primary rat hepatocytes, as LDH is ubiquitously expressed and exclusively degraded by autophagy (Kopitz et al. 1990). The benefit of the LDH sequestration assay was the ability to measure sequestration activity of endogenous cargo in a highly quantitative manner, allowing for the assessment of the formation of sealed autophagosomes as opposed to the limited possibility of that using TEM. The whole process of autophagy does not only rely on the initial sequestration of cargo, but also fusion with lysosomes and cargo degradation. The LLPD assay provides information on that account, measuring the degradation of radiolabeled long-lived proteins, as short-lived proteins are excluded in a chase step (Luhr et al. 2018). Both the LDH sequestration and LLPD assay rely on inhibiting lysosomal proteolysis to measure autophagic activity. This may obscure results, as it precludes the evaluation of lysosomal function and thus the complete assessment of autophagy (Mizushima et al. 2010, Klionsky et al. 2021). As we were studying NPs that need to be internalized in this work, we were eager to find additional methods of studying autophagic activity that does not rely on lysosomal inhibitors as this may affect the routing of cargo to the lysosomes. Our attention turned to mKeima-based assays, a pH-responsive fluorescent protein resistant to lysosomal proteases, thus providing a cumulative readout of autophagic activity as it stably accumulates in the lysosomes (Katayama et al. 2011). The major advantage of using mKeima as a fluorophore is the lack of requirement of additional inhibitors to quantify autophagic activity. By combining the autophagic LDH sequestration assay with the LDHB-mKeima, free mKeima and LLPD degradation assay we can distinguish whether an inhibition in degradation is a result of inhibited autophagosome biogenesis, inhibited fusion with lysosomes or inhibited lysosomal activity. The coherence between the different functional autophagy assays were put to the test by our investigation into the effect of PBCA treatment in Paper III. Strikingly, even though each assay accounts for a slightly different aspect of the autophagy pathway, the agreement between the results with the various PBCA concentrations was highly accurate - strengthening the independent use of each assay.

The PACA-induced type and magnitude of cellular stress affect nanoparticle-induced mode of cell death

POCA-induced apoptotic cell death

Treatment with the POCA NP seems to elicit a more severe and acute effect on cells, resulting in the highest cytotoxicity of the three PACA variants and a strong caspase activation at far lower concentrations than PEBCA and PBCA (Paper I). Furthermore, the POCA-induced ER stress and cytotoxicity seem independent of oxidative stress (Paper I) and lead to an inhibition in autophagic cargo degradation (Paper II). ER is a crucial organelle for the cell, and excessive ER stress has been shown to trigger apoptosis. The same ER sensors involved in reestablishing full function is also involved in pro-death signaling upon severe or prolonged ER stress (Rutkowski et al. 2006, Walter et al. 2011).

IRE1 is described to be a pro-apoptotic pathway via interaction with TRAF2 and activation of JNK (Urano et al. 2000, Nishitoh et al. 2002), as well as degrading anti-apoptotic mRNAs through RIDD (Bouchecareilh et al. 2011). Furthermore, the transcription factor CHOP is described to link PERK activation to apoptosis (Urra et al. 2013), especially through upregulation of the pro-apoptotic Bcl2 family. Bcl2 family proteins are involved in cytochrome c release from the mitochondria and subsequent activation of caspase-9 and the downstream caspases-3 and -7, executing cell death (Urra et al. 2013, Hetz et al. 2018). However, CHOP-dependent ER stress-induced cell death have also been linked to 'death receptor 5' (He et al. 2002, Yamaguchi et al. 2004, Lu et al. 2014). Several NPs are demonstrated to activate apoptosis downstream of ER stress. Silver NPs are demonstrated to rapidly activate the three ER sensors, PERK, IRE1 and ATF6, and induce UPR-dependent apoptosis in cancerous cells (Simard et al. 2016). Gold NPs were on the other hand found to induce ER stress by inhibiting ERAD, and subsequently activate apoptosis through ER stress-induced activation of PERK-ATF4-CHOP pathway and oxidative stress (Guofang et al. 2021). Moreover, silicon dioxide NPs were found to induce ER- and mitochondrial stress through ROS increased PI3K activity and AKT phosphorylation ultimately initiating apoptosis (Lee et al. 2020). Treatment with the POCA NP potently accumulate ATF4 and CHOP downstream of PERK activation (Paper I). Hence, it seems likely that the POCA induced insult was too severe for the cell to regain ER homeostasis, and that these effectors were involved in pro-death signaling upon POCA treatment instead.

PBCA- and PEBCA-induced ferroptotic cell death

Where the POCA particle selectively caused apoptotic cell death, not rescuable by co-treatment with antioxidants, the PBCA particle selectively caused ferroptotic cell death not rescuable by the pan-caspase inhibitor z-VAD. Even the less cytotoxic PEBCA particle potently induced cell death in a manner rescued by ferroptosis inhibitors in cystine-free medium. Resultantly, ferroptosis was initiated by PBCA and PEBCA depending on the cell's ability to mount a redox defense. Upregulation of ATF4 and Nrf2 by the two NPs act as pro-survival pathways protecting the cells from ferroptosis. Nevertheless, PEBCA was far less toxic than PBCA over the panel of cell lines, and our data point to a more potent and prolonged Nrf2-mediated antioxidant response induced by PEBCA as the cause of the lower cytotoxicity from this particle.

Ferroptosis is a non-apoptotic form of regulated cell death associated with accumulation of ROS in an iron-dependent manner, depletion of GSH and excessive lipid peroxidation (Dixon et al. 2012, Stockwell et al. 2017, Lu et al. 2018). Where apoptosis is often anti-inflammatory and immune-silent, ferroptosis is pro-inflammatory due to release of damage-associated molecular pattern molecules (Linkermann et al. 2014, Kim et al. 2016, Galluzzi et al. 2018). Loss of glutathione peroxidase 4 (GPX4) activity initiates ferroptosis through two distinct mechanisms, one direct and one indirect. In the indirect mechanism inhibition of system X_C^-, the cystine/glutamate antiporter (Bridges et al. 2012), reduces the intracellular level of cystine and consequently cysteine, the precursor of GSH. This leads to GSH depletion and subsequent inactivation of GPX4, as GSH is an essential cofactor for the GPX4 phospholipid peroxidase activity to catalyze the reduction of lipid peroxides. Thus, inhibition of system X_C^- by small molecules such as erastin (Dolma et al. 2003) leads to accumulation of lethal lipid peroxides and initiation of ferroptosis (Dixon et al. 2012, Dixon et al. 2014, Yang et al. 2014). Notably, also inhibition of the GSH synthesis is sufficient to initiate ferroptosis (Yang et al. 2014). In the direct mechanism, GPX4 is inhibited causing lipid ROS and ferroptotic cell death. This can be achieved by loss of GPX4 activity, such as for (1S, 3R)-RSL3 which covalently target the GPX4 active site selenocysteine in an irreversible manner (Yang et al. 2014, Yang et al. 2016), or by increased degradation, such as for FIN56 decreasing GPX4 abundance (Shimada et al. 2016). As such, ferroptosis can be inhibited by small-molecule lipophilic antioxidants like ferrostatin-1 and liproxstatin-1, and iron chelators like deferiprone, whereas inhibitors of other cell death mechanisms like z-VAD cannot.

Activation of cell death is a key strategy for cancer therapy, however development of drug resistance to existing chemotherapeutics remains a problem. Ferroptosis-inducing compounds have been shown to suppress cancer growth and progression (Wu et al. 2020), and act synergistically with chemotherapeutics such as cisplatin (Roh et al. 2016, Sato et al. 2018), temozolomide (Chen et al. 2015), cytarabine/ara-C and doxorubicin/Adriamycin (Narang et al. 2007, Yu et al. 2015). A high-mesenchymal cell state is associated with the most drug resistant cancer cells (Zhang et al. 2018). These cells have been found to depend on the lipid-peroxidase pathway, and GPX4 specifically, to protect against ferroptosis (Hangauer et al. 2017, Viswanathan et al. 2017). Furthermore, oncogenic mutations are described to show greater sensitivity to cystine deprivation- or erastin-induced ferroptosis (Yagoda et al. 2007, Yang et al. 2008, Poursaitidis et al. 2017). Ferroptosis-sensitive cells are highly vulnerable to GSH depletion and depend on constant import of the GSH precursor cystine (Lu et al. 2018). In accordance with the discussion of the cystine transporter being a target to reduce drug resistance (Gout et al. 2003), ferroptosis-inducing compounds have emerged as a promising treatment option for the drug-resistant highly mesenchymal cancer cells containing this metabolic signature (Bebber et al. 2020, Wu et al. 2020).

Identification of ferroptosis-inducing NPs, such as PBCA and PEBCA, is of considerable interest in drug-delivery to increase treatment outcome; as a drug carrier by sensitizing the target cell or to act as sole treatment modality (Liu et al. 2018, Wang et al. 2018, Qian et al. 2019, Shan et al. 2020). In such a scenario, PBCA might be an especially suited NP drug carrier candidate to induce ferroptosis in hard-to-kill drug-resistant cancer cells compared to PEBCA. The pro-survival responses induced by PEBCA may comprise treatment outcome, as upregulated Nrf2 and SLC7A11 has been demonstrated to increase ferroptosis resistance (Ju et al. 2015, Sun et al. 2016). PEBCA demonstrate only minor induction of ferroptosis in MDA-MB-231 cells when given as sole treatment. However, upon deprivation of cystine, PEBCA induce ferroptosis at similar levels as the more potent ferroptosis-inducer PBCA. Such a potent induction of ferroptosis under amino acid deprivation has also been demonstrated for PEG-coated silica NPs functionalized with a melanoma-targeting peptide (αMSH-PEG-C′ dots) that are otherwise well tolerated by cells (Kim et al. 2016). They found that combined treatment of a variety of different cancer cell types with αMSH-PEG-C′ dots and amino acid starvation can synergize to induce ferroptosis. One cancer cell type, HT-1080 fibrosarcoma cells, was found to be particularly sensitive to particle treatment and underwent ferroptosis in full media and at tenfold lower concentrations in starvation media. They further found that high-dose delivery of

αMSH-PEG-C′ dots can inhibit tumor growth and cause tumor regression to a greater extent in HT-1080 (particle sensitive cells) than renal carcinoma 786-O xenografts, although both demonstrated reduction in tumor growth compared to control (Kim et al. 2016). This indicates that particles such as PEBCA and αMSH-PEG-C′ dots, which are well tolerated by many cell types, can be exploited for enhanced treatment outcome by ferroptosis induction in particularly sensitive cancer cells.

From our investigations in Paper I, we know that PEBCA and PBCA treated MDA-MB-231 cells rely on the ATF4-Nrf2-SLC7A11 pathway for increased antioxidant response and survival, and that both PEBCA and PBCA cause substantial depletion of GSH and increase in lipid peroxidation upon cystine deprivation. αMSH-PEG-C′ dots are suggested to induce such a lethal metabolic imbalance executing ferroptosis by increasing intracellular delivery of iron due to iron loading of the αMSH-PEG-C′ dots in culture media, which during amino acid starvation becomes detrimental as increased iron uptake lead to depletion of GSH (Kim et al. 2016). Overloading of labile iron, Fe^{2+}, is capable of directly catalyzing lipid peroxidation via Fenton reactions and ultimately lead to ferroptosis (Lu et al. 2018). We have not identified the exact mechanism of GSH depletion by PEBCA or PBCA and can only speculate whether such an iron loading can occur for these particles or whether the particle properties are facilitating electron transfer causing GSH depletion or free radical production. Moreover, ferroptosis has been shown to be induced by tungsten disulfide and Molybdenum disulfide nanosheets in epithelial (BEAS-2B) and macrophage (THP-1) cells by suppression of GPX4, oxygen radical formation and lipid peroxidation. This was not caused by particle dissolution, but rather by particle-increased lysosomal iron release due to the strong oxidation capacity and phospholipid-binding affinity of unsaturated metal atoms on the particle surface (Xu et al. 2020).

Metal-based NPs are known to cause oxidative stress through Fenton or Fenton-like reactions (Nel et al. 2006, He et al. 2012, Tian et al. 2018). As such, these NPs are particularly studied to enhance cancer treatment by triggering ferroptosis alone or in combination with a cargo treatment through the intracellular release of Fe^{2+} upon degradation in lysosomes. Some examples are the exploration of doxorubicin-loaded ferritin NPs (Yang et al. 2019), Fe^{2+}-based metal organic frameworks (He et al. 2020, Wan et al. 2020, Xu et al. 2020), Fenton-reaction-acceleratable magnetic NPs (Fe_3O_4) simultaneously increasing Fe^{2+}, Fe^{3+} and H_2O_2 (Shen et al. 2018), or sulfasalazine-loaded mesoporous magnetic NPs for the simultaneous inhibition of cystine uptake and Fe^{2+} release (Jiang et al. 2020). Moreover, various non-metal-based NP carriers are also exploited to deliver cargo molecules aimed at inducing ferroptosis to sensitize

targeted cells for treatment effect. Guo et al., 2020, designed hypoxia-responsive polymer micelles encapsulating the GPX4 inhibitor RLS3, to enhance the potency of the ferroptotic inducer in solid tumors. The micelles were PEG-coated through an azobenzene linker, which allows shedding of the PEG coat under hypoxic conditions and enhanced uptake of the RLS3-loaded micelles in the murine breast cancer cell line 4T1 (Guo et al. 2020). Chen et al., 2019, exploited the property of polydopamine to chelate Fe^{2+} and Fe^{3+} and create ultrasmall PEG-coated polydopamine NPs with a pH-dependent release of Fe^{2+} or Fe^{3+} ions. The particles were demonstrated to exhibit effective anticancer effect on 4T1 and U87MG cell lines, as well as tumor-bearing Balb/c mice through promoting ferroptosis (Chen et al. 2019). In such a manner PBCA and PEBCA NPs may be optimal as carriers of ferroptosis-inducers, synergizing the induction of ferroptotic cell death.

Ferritin is the major intracellular iron storage complex (Theil 2004). Oncogenic RAS signaling have been implicated to downregulate ferritin to increase the intracellular labile iron pool to replenish proliferation-driving enzymes with sufficient iron to function (Yang et al. 2008). This may contribute to explain why some cancer types are particularly sensitive to disturbances in iron/redox homeostasis. Excessive iron may participate in Fenton reactions increasing ROS and lipid peroxidation, promoting ferroptosis. Corroborating the contribution by labile iron, iron chelators inhibits erastin-mediated ferroptosis, whereas exogenous supply of iron enhances erastin-mediated ferroptosis (Yang et al. 2008, Dixon et al. 2012).

Autophagy have been demonstrated to contribute to the early stages of ferroptosis by NCOA4-mediated *ferritinophagy*, the selective autophagic degradation of ferritin (Dowdle et al. 2014, Mancias et al. 2014), supplying labile iron (Hou et al. 2016). NCOA4-overexpression increased the levels of Fe^{2+} and malondialdehyde (MDA, product of lipid peroxidation) and reduced the levels of GSH, whereas NCOA4-depletion reduced the levels of Fe^{2+} and MDA and increased the levels of GSH. Indicating that NCOA4-mediated ferritin degradation is at the crossroad of redox metabolism in ferroptosis (Hou et al. 2016). In accordance with this, inhibition of lysosomal function or knockdown of key autophagy players can significantly attenuate and delay the ferroptosis process (Gao et al. 2016).

In Paper III, we find that PBCA at concentrations below 12.5 μg/ml can increase autophagic degradation in RPE-1 cells through MAPK signal pathway activation. PBCA at 12.5 μg/ml was also shown to cause a rapid depletion of GSH and a later accumulation of intracellular ROS. In Paper I, we show that PBCA can induce ferroptosis in MDA-MB-231

cells, a triple-negative breast cancer cell line with a high mesenchymal state, fitting the category of ferroptosis-sensitive cancer cells. The induction of ferroptosis was attributed to a rapid increase in ROS and depletion of GSH, and a later accumulation of lipid peroxidation in MDA-MB-231 cells. In Paper II, we show that PBCA increase *LC3B* mRNA without an effect on increased LC3-II protein levels in MDA-MB-231. The lack of increased LC3-II can be caused by the additional effect of oxidative stress-induced inhibition in LC3 lipidation as confirmed in RPE-1 cells in Paper III, and described as a mechanism by Frudd et al., 2018, (Frudd et al. 2018). As we have not assessed the possibility of lower concentrations of PBCA stimulating autophagic degradation in MDA-MB-231 cells, we can only speculate whether increased autophagic degradation at an early stage in PBCA treatment is promoting ferroptosis in this cell line based on the findings in RPE-1 cells in Paper III. Conversely, we have not investigated whether the increased bulk degradation observed in RPE-1 cells is increasing the intracellular labile iron pool, and whether this is contributing to or causing the oxidative stress measured, and whether this can ultimately initiate ferroptosis in RPE-1 cells. Furthermore, it would be interesting to investigate whether PBCA treatment impacts ferritinophagy specifically and can be exploited to promote ferroptosis in ferroptosis-sensitive cells or in combination with ferroptosis-promoting conditions such as cystine deprivation. Moreover, ferritin degradation has also been shown to be independent of canonical autophagy (Goodwin et al. 2017), or mediated by ESCRT-III (endosomal sorting complex required for transport III)-dependent endosomal microautophagy during the immediate response to amino acid starvation (Mejlvang et al. 2018). Such a mechanism for ferritin degradation can possibly be targeted by NPs as they are primary internalized by endocytosis and may cause endosomal damage recruiting ESCRT-III (Radulovic et al. 2018).

More interestingly, the MAPK signaling pathway, consisting of JNK, p38 and ERK, has been implicated in ferroptosis. Inhibition of MEK1/2 (upstream of ERK1/2) attenuates erastin-induced lethality in Ras-mutated cancer cells, indicating that the activated RAS-RAF-MEK pathway renders the cells more sensitive to erastin-induced ferroptosis. They further demonstrate that there is a modest correlation between ERK1/2 phosphorylation status and erastin sensitivity in 12 sarcoma cell lines (Yagoda et al. 2007). Moreover, erastin is found to phosphorylate and activate p38 and JNK in acute myeloid leukemia HL-60 cells, and inhibition of these kinases or knockdown of p38α inhibit erastin-induced cell death (Yu et al. 2015). In Paper III we demonstrate potent activation of the three MAPKs p38, JNK and ERK by treatment with PBCA in RPE-1 cells. We further demonstrate that p38 and JNK activation is involved in

the stimulated autophagic degradation induced by PBCA, most likely by phosphorylating Beclin 1 S90, potentially increasing the Vps34 lipid kinase activity. Moreover, Beclin 1 has been demonstrated to play a non-autophagic role in promoting ferroptosis by directly binding SLC7A11 and blocking system X_C^-, the cystine/glutamate antiporter activity (Song et al. 2018, Guo et al. 2019). Inhibitors of system X_C^-, such as erastin and sulfasalazine, was shown to induce AMPK-mediated phosphorylation of Beclin 1 S90/93/96 that was required for the formation of Beclin 1-SLC7A11 complex and subsequently promote GSH depletion and lipid peroxidation (Song et al. 2018). AMPK-mediated phosphorylation of Beclin 1 S93/96 during glucose starvation (Kim et al. 2013) and MAPK-mediated phosphorylation of Beclin 1 S90/93 during amino acid starvation (Fogel et al. 2013, Wei et al. 2015) have also been shown to be essential for activating the PI3KC3 complex to induce autophagy. However, corroborating the non-autophagic role of Beclin 1 in promoting ferroptosis, iron accumulation was not affected by the formation of Beclin 1-SLC7A11 complex inhibiting system X_C^- activity (Song et al. 2018). It is interesting to speculate whether the MAPK activation found to contribute to erastin-induced ferroptosis also acts through formation of Beclin 1-SLC7A11 complex downstream of Beclin 1 S90/93/96 phosphorylation. Although, we do not observe AMPK activation by PBCA treatment in RPE-1 cells in Paper III, we cannot exclude that AMPK activation or MAPK activation is involved in the PBCA-mediated ferroptosis induction seen in MDA-MB-231 cells in Paper I. This makes PBCA a particularly interesting NP candidate for carrying ferroptosis-inducers such as erastin, as the particle itself may contribute by enhancing MAPK activation and possibly increasing the intracellular labile iron pool promoting ferroptosis.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In conclusion, it is evident through this work that NPs are highly specific entities of which general conclusions are not easily made. It seems their exact composition dictate their exact biological identity affecting how they interact with cells and what stress they cause for the cells. Furthermore, cells are also highly adapted to the stress they have evolved to handle, and consequently the same NP may not induce the same level of stress or cytotoxicity over a variation of cell lines. In this work we have demonstrated the value of in-depth *in vitro* cell culture studies to investigate NP-induced stress and the outcome of the NP-cell interaction. POCA consistently induced ER stress at a level highly cytotoxic in our panel of cell lines, although with some variation. The severity of the insult seemed to induce apoptosis without engaging pro-survival responses. Further supporting this, POCA accumulated LC3-II as an effect of inhibited fusion with the lysosomes and hence reduced autophagic LLPD as opposed to increased cytoprotective autophagy. PEBCA consistently induced very low cytotoxicity over the panel of cell lines. This was attributed to a potent pro-survival accumulation of ATF4 and more importantly Nrf2, upregulating key players in the antioxidant response. Of significant importance was the cystine transporter, SLC7A11, inhibiting propagation of ROS and ferroptosis until the redox balance was additionally challenged by cystine starvation. Furthermore, ATF4 was found to cause transcriptional upregulation of *LC3B* mRNA leading to an increased LC3-II protein level maintaining autophagy at normal/basal levels throughout the stressful situation. PBCA was consistently the mediocre NP of the three, inducing cytotoxicity downstream of oxidative stress. Although PBCA also induced pro-survival ATF4 and Nrf2 signaling the response was too weak, or rather the insult too strong, to inhibit ROS propagation, GSH depletion and ferroptosis. Interestingly, the redox imbalance induced by PBCA seemed to cause three distinct autophagy phenotypes, reflected in the cytotoxic dose-response curves. At the lowest concentrations (3.12 and 6.25 µg/ml), the redox imbalance increased autophagic cargo degradation in a manner dependent on JNK and p38 MAPK activation. At these concentrations, cytotoxicity of PBCA was low and the combined efforts of the antioxidant response, upregulating SLC7A11, and increased autophagy maintains cellular homeostasis. Treatment with 12.5 µg/ml PBCA, was observed to be the turning point where GSH most likely is depleted to such an extent that it can no longer protect the ATGs involved in LC3 conjugation from oxidative modification. Consequently, LC3 lipidation is reduced, but cytoprotective autophagy is increased in the fight to regain homeostasis. At higher concentrations (>12.5

μg/ml) the oxidative insult or rather redox imbalance is too severe. The inhibition in LC3 lipidation is more pronounced and the cells are unable to sustain autophagy, dramatically reducing autophagic cargo degradation as cytotoxicity increase.

ROS is a common underlying mechanism of NP-induced cytotoxicity for a variety of NPs (Wen et al. 2020). In this work we have deciphered several mechanisms as to how NP-induced ROS may regulate autophagy from the transcriptional level to actual cargo degradation. Particularly interesting would be to follow up the inhibition in LC3 lipidation observed for PBCA, and whether this is a mechanism by which other NPs ultimately inhibit autophagic degradation. Moreover, to specifically follow up the phenotype observed at 12.5 μg/ml PBCA, where LC3 lipidation is inhibited without a concomitant reduction in autophagic turnover. Also the autophagy stimulation observed at lower concentrations of PBCA would be interesting to follow up. Can other ROS-inducing NPs cause activation of autophagy through MAPK activation? It would also be of interest to elucidate in more depth how MAPK activation and Beclin 1 S90 phosphorylation is able to increase autophagic degradation beyond that induced by the mTOR inhibitor Torin1. It would further be interesting to follow up whether the observed increase in bulk autophagic degradation is reflected as an increase in selective autophagy by use of various selective mKeima reporters. Of particular interest would be to investigate ER-phagy, aggrephagy and mitophagy downstream of NP-induced oxidative stress and/or ER stress, as well as the possible impact of ferritinophagy on ferroptosis induction by NPs.

By deciphering how various NPs induce stress and the specific mechanisms at play, one can identify specific NP drug carriers compatible with specific treatment outcomes. As illustrated through this work, a strong pro-survival upregulation of Nrf2 and SLC7A11 may increase drug resistance in the target cells (Okuno et al. 2003, Huang et al. 2005) and as such can compromise cancer treatment, whereas ferroptosis-induction through increased release of labile iron, GSH depletion or increased ROS/lipid peroxidation may suppress cancer growth of the drug-resistant highly mesenchymal cancer cells (Zhang et al. 2018, Wu et al. 2020). Through this work we have gathered a platform of experiments to screen the cellular stress responses initiated by various NPs. By using the mKeima-based system we can identify NPs that stimulate autophagic degradation, which may be beneficial for neurodegenerative disorders (Towers et al. 2016). Particularly as impaired autophagy has been found to promote exocytosis of α-synuclein (Poehler et al. 2014). Increased exocytosis may contribute to reduced aggregate load on the cellular level, phenotypically mimicking increased degradation, but may contribute to increased spreading of the toxic aggregates as they are taken up by neighboring cells.

It is now generally acknowledged that characterization of the protein corona forming around the NP is a first step towards understanding the nature of NP-mediated biological effects. Several methods are available for studying the variation in protein corona (Zeng et al. 2019), and even the contribution from the weakly interacting proteins of the soft corona (Mohammad-Beigi et al. 2020). In this work we hypothesize that variations in the protein corona forming around the three highly similar PACA NPs are involved in eliciting the variation in cellular stress responses, having consequences for cytotoxicity and mode of cell death. Hence, it would be interesting to characterize the protein corona of the three PACA variants and seek answers to what might be dictating such a varied response.

A major point that would be highly interesting and important to address, is how the induced stress responses relate to an effect at the level of the organism? Can the effects observed by the PACA variants be reproduced in an organism, and hence exploited in a treatment setting? The route of NP delivery may change the NP coat and hence how the particles interact with the cells and where the particles accumulate. *In vitro* studies do not fully simulate the highly dynamic nature of extracellular environments, and the protein corona have been found to be influenced by circulation (Pozzi et al. 2015, Palchetti et al. 2017). Furthermore, protein coronas formed in “cell-conditioned” media and “unconditioned” media induce differences in pro-inflammatory cytokine secretion and immune cell apoptosis (Dai et al. 2017).

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