Smart use of the tumor microenvironment to enhance the therapeutic efficiency of anti-cancer nanomedicines

Layman´s summary

Most patient with cancer are treated with chemotherapy, radiotherapy and surgery. However, these treatments cause damage to healthy cells as well, causing several side effects. To reduce the side effect, more cancer specific therapies are developed, including nanomedicines.

In nanomedicines, materials with a size smaller than 1 µm are used for medical applications like diagnosis and treatment. Nanomedicines show some advantages over conventional treatment, including less side effects due to accumulation in the tumor. Since nanomedicines are small in size and blood vessels in the tumor have small gaps, the nanomedicine can leak out of the blood vessel into the tumor. Blood vessels in healthy tissue are not leaky, therefore the accumulation of the nanomedicine only occurs in the tumor. Thereby minimizing the damage to healthy tissue and the subsequent side effects.

Although, the nanomedicine have several advantages, treatment with nanomedicines show lack in improvement of patient outcome. The patient outcome can be enhanced by adjusting the features of the nanomedicines. However, the adjustments that increase the number of nanomedicines that reaches the tumor are contradictory to the adjustments that are needed to reach all areas of the tumor. To overcome this problem, nanomedicines are designed to change their feature after reaching the tumor, e.g. the nanomedicines decreases in size after entering the tumor to reach more dense areas of the tumor. To allow for this change, the nanomedicines are generated in such a way that they are sensitive to specific characteristics of the tumor, for example a slightly lower pH compared to healthy tissue. In reaction to the slightly lower pH, the nanomedicines undergo a change, such as reduction in size. This enables a nanomedicines to have features which are important for both accumulating in the tumor and reaching all areas of the tumor. The discussed nanomedicines showed promising results, which might result in improved therapeutic outcome.

Abstract

Nanomedicines are a promising type of therapy for solid cancer and are characterized by their submicron size. The nanomedicines show some advantages over conventional treatment, for example they can passively accumulate in the tumor due to the small size of the nanomedicine and the EPR effect of the tumor. These advantages resulted in the first FDA approved nanomedicine for cancer therapy in 1995. More nanomedicines followed in the subsequent years.

Unfortunately, these nanomedicines showed limited therapeutics efficiency. The therapeutic efficiency could be approved by increasing the circulation time of the nanoparticle, enhancing the accumulation in the tumor or enhancing the penetration to deep and hypoxic areas of the tumor. The nanoparticles can be adjusted to increase the therapeutic outcome. A nanoparticle with a hydrophilic feature, size of 100-200 nm and negative or neutral charge are essential for prolonged circulation time and accumulation in the tumor via the EPR effect. While a small size and positive charge are necessary for deep tumor penetration and tumor cell internalization.

Different studies show the possibilities to use the tumor microenvironment to switch the feature of a nanomedicine to enable for both enhances circulation time and deep tumor penetration. Nanoparticles can change in hydrophobicity as result of the acidic environment of the tumor. In other studies it was shown, that the acidic environment or the elevated levels of matrix metalloproteases could be used to reduce the size of a nanomedicine after reaching the tumor. In addition, the high levels of glutathione and reductases were utilized to prevent drug leakage and reach hypoxic areas of the tumor. The different TME-sensitive nanomedicines showed promising results with enhanced therapeutic efficiency.

Introduction

Cancer is one of the leading cause of death worldwide, contributing to nearly 10 million deaths in 2020[1]. Surgery, chemotherapy and radiation are continued to be used as the first line therapy for cancer. However, the aspecific targeting results in damage to healthy cells causing numerous side-effects. To overcome the lack of specificity, more specific cancer therapies made their entrances during the last years. One promising type of therapy for solid cancer is nanomedicines[2,3]. Nanomedicines are characterized by their submicron size. In principle being smaller than 1 µm, however in practice most nanomedicines are around 100 nm in size. They are used in medical applications like diagnosis or treatment[3]. They can be composed of diverse materials like lipids, polymers, metal or silica, or can be virus- or antibody-based.

Advantages of nanomedicines

Nanomedicines offer several advantages over conventional treatments. Firstly, the use of nanoparticles improves tumor accumulation due to prolonged circulation time compared to free drugs. The circulation time is increased by limited leakage in healthy tissue and less clearance from the blood stream. The size of the nanoparticles ensure that it is difficult to squeeze out of the blood stream through the tightly packed endothelial cells. When there is limited leakage of the drug, it prolongs the circulation time.

In addition, the increased size of the nanoparticles reduced the clearance from the blood by the kidneys[5]. In addition, the nanoparticles can be further designed to prevent clearance from the blood, for example by coating the nanoparticles with polyethylene glycol (PEG)[6]. The reduced clearance from the blood, further enhanced the circulation time in the blood.

Secondly, nanomedicines enable the use of hydrophobic drugs. Most of the anticancer drugs, including the commonly used doxorubicin and paclitaxel, are hydrophobic drugs. These drugs have a low solubility in water, which makes it difficult to dissolve in the hydrophilic blood, which impairs transport through the body[7].

This poor solubility can be overcome by the use of nanoparticles, such as micelles. Micelles composes of amphiphilic molecules, molecules with a hydrophobic and hydrophilic part, and are formed through self-assembly in presence of an aqueous solution. The hydrophilic parts point towards the outside of the micelle, while all hydrophobic parts are in the core[8]. Micelles enable the option to use hydrophobic drugs, since the hydrophobic drugs can be transported in the core of the micelle, while the outside it hydrophilic. Without the micelle, the hydrophobic drug would have a low bioavailability due to their minimal solubility in water[9].

Lastly, nanomedicines can accumulate in the tumor via a passive targeting mechanism. There is no specific ligand attached to the nanomedicine, the nanomedicine passively accumulate in the tumors due to enhanced permeability and retention (EPR) effect in solid tumors[10,11]. The EPR effect is characteristic for tumor tissue and is a results of the leaky blood vessels and impaired lymphatic drainage in the tumor.

Tumor cells have a high demand of oxygen and nutrients. To supply for this high demand, tumors grow their own tumor vessels, a process known as angiogenesis. However, these newly formed vessels are different than blood vessels in healthy tissue. Blood vessels in healthy tissue have regular shapes and the endothelial cells are closely connected by tight junction[12]. In contrast, the blood vessels in tumor tissue are characterized by an irregular shape and have gaps of 100-790 nm between the endothelial cells (Figure 1)[13]. As a result of these leaky vessels, tumors have enhanced permeability, which allows accumulation of nanomedicines inside the tumor.

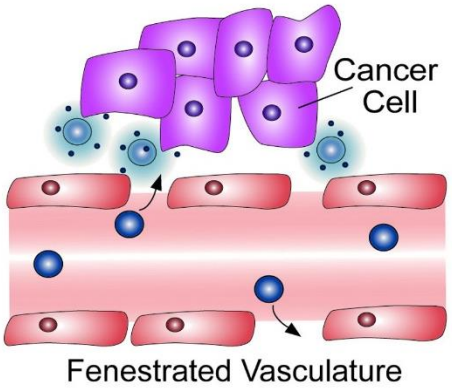

Figure 1: Fenestrated vasculature of the tumor. The blood vessels in the tumor tissue have gaps of 100-780 nm between the endothelial cells, enabling the leakage of nanomedicine out of the blood stream into the tumor. Resulting in the passive targeting of tumor cells[13].

Inside the tumor tissue, the nanomedicines also have an increased retention time due to the poor lymphatic drainage. Normally, fluid or nanoparticles that leak out of the tissue will be drained back into the circulation by the lymphatic system[14]. However, the lymphatic drainage in tumors is impaired resulting in the longer retention time.

Since the EPR effect is a characteristics of the tumor, the anticancer nanoparticles accumulate in the tumor tissue compared to the healthy tissue thereby enhancing the delivery to the tumor site and minimizing the exposure to healthy cells. The anticancer nanomedicine accumulate and retain in the tumor due to the EPR effect.

Since nanoparticles improve the circulation time, enable the use of hydrophobic drugs and passively accumulate in the tumor tissue due to the EPR effect, it is not surprising that nanoparticles made its way to the clinic.

Doxil, the first FDA approved nanomedicine

In 1995, the first nanomedicine for cancer therapy was FDA approved, which was a liposomal Doxorubicin called Doxil. Doxorubicin, a chemotherapy drug, was encapsulated in a liposome of 80-90 nm, which was coated with polyethylene glycol (PEG) to stabilize the liposome. A liposome is a lipid bilayer and is able to carrier hydrophobic and hydrophobic anti-cancer drugs[15]. The use of liposomes resulted in less side effect on healthy cells compared to doxorubicin alone. In addition, the liposome carrier increased the circulated time and thereby improving the pharmacokinetic profile of doxorubicin.

Although Doxil showed a better pharmacokinetic profile, an increase in patient survival was not observed. After the first nanomedicine FDA approve, more nanomedicines followed in the subsequent years. Some of these new developed nanomedicines are based on a different type of nanocarrier such as micelles or nanospheres[16].

The lack of improvement in patient outcome might be explained by low delivery efficacy of the nanomedicine. The low delivery efficacy is caused by the many challenges nanomedicine face during the delivery of the drug to the tumor cells, including blood circulation clearance, poor tumor accumulation, inefficient tumor penetration and intracellular delivery[17].

The required characteristics of the nanomedicines to overcome the challenges in drug delivery

Nanomedicines can be cleared from the system circulation by the kidneys or the mononuclear phagocyte system. During renal clearance, nanomedicines are excreted into the urine by the kidneys.

Renal clearance can be prevented by increasing the size of the nanomedicine since small particles are rapidly cleared from the blood by the kidneys[18].

In addition, nanoparticles can also be cleared by phagocytes. The nanoparticles become covered with non-specific proteins in the blood circulation, a process called opsonization, to mark them for elimination by phagocytes. Opsonization of nanoparticles is mostly established by electrostatic and hydrophobic interactions between the nanoparticles and opsonin proteins. Neutrally and negatively charged nanoparticles are less eligible for opsonization compared to positively charged particles. Therefore, negatively and neutrally charged nanoparticles show longer circulation half-lives[19]. The same applies for hydrophilic nanoparticles, which are less cleared from the blood compared to hydrophobic particles[20]. Therefore, neutrally or negatively charged, hydrophilic nanoparticles would be preferred to prolong circulation time.

When the nanoparticles have dodged the circulatory clearance and arrive at the tumor site, the next challenges for the nanoparticles are accumulation in the tumor and penetration through the tumor tissue. As previously described, nanoparticles accumulated in the tumor as a result of the EPR effect. However, accumulation of a nanomedicine in the tumor is not sufficient for a high therapeutic efficacy. A nanoparticles with a size of around 100-200 nm show prolonged blood circulation and have more chance to accumulate in the tumor as a result of the EPR effect[21,22,23]. However, large particle size show limited penetration into the tumor due to the dense extracellular matrix (ECM), a matrix of proteins such as proteoglycans, glycoproteins, elastin, fibronectins and collagen, in the tumors[17,24]. The ECM impairs the efficacy of the anti-cancer drugs since it forms a physical barrier between the drug and tumor cells. Nanoparticles bigger than the mesh size of the ECM cannot penetrate through the ECM and get trapped. Therefore, nanoparticles should be small enough to diffuse through the tumor[25].

The last step of the drug delivery is uptake by the tumor cells. Different studies have demonstrated that positively charged nanoparticles are better internalized by cells than neutrally of negatively charged nanoparticles due to the negative charge of the cell membrane[19,26]. In addition, Wang, H. *et al.* demonstrated that positively charged nanoparticles have a 2.5-fold higher accumulation in tumor cells due to better tumor penetration and cellular uptake compared to negatively or neutrally charged particles[19]. Moreover, the cell membrane is mainly permeable to hydrophobic molecules, therefore a hydrophilic nanoparticle would complicate the uptake by tumor cells. Hydrophobic nanomedicines would be favoured for cellular uptake[26].

Nanoparticles require different characteristics for long circulation time and enhanced tumor accumulation than for deep tumor penetration and cellular uptake. It would be desirable to have a hydrophilic and neutrally or negatively charged nanoparticle with a size of 100-200 nm in the circulation, which changes to hydrophobic and positively charged in the tumor to enhance tumor penetration and cellular uptake.

Altogether, the nanomedicines face several challenges from injection till cellular uptake, which results in low therapeutic efficiency. To enhance the therapeutic efficiency, the nanomedicine should be adapted to improve the tumor accumulation, penetration and cellular uptake. Interestingly, nanoparticles require different features to improve circulation time and tumor accumulation than to enhance tumor penetration and cellular uptake. On one hand, neutral or negative charge, hydrophilicity and relatively large size are desirable for long circulation and tumor accumulation. While on the other hand, positive charge, hydrophobicity and small size are important for deep tumor penetration and cellular uptake. To meet both requirements, the characteristics of nanomedicines should alter after tumor accumulation. The tumor microenvironment could be used as internal

stimulus since the TME has specific physiological features compared to normal tissue, which enables changes in nanomedicine characteristics after tumor accumulation[21].

Specific characteristics of the tumor microenvironment

Cancer is characterized, among other things, by uncontrolled proliferation of cancer cells, invasion and metastasis into tissue and sustained angiogenesis[27]. All these hallmarks of cancer affect the biochemistry and physiology around the tumor cells and this specific area around the tumor can be distinguished as the tumor microenvironment (TME). The TME consist of tumor cells, immune cells, disorganized blood vessels and tumor extra cellular matrix (ECM), and is characterized by several elements including elevated levels of certain enzymes, slightly acidic environment, hypoxia areas, overexpression of reductases and increased levels of GSH[17].

Extra cellular matrix

The is the largest component of the TME is the ECM, which is a matrix of proteins such as proteoglycans, glycoproteins, elastin, fibronectins and collagen. A large part of the tumor, around 60%, consists of the ECM. The ECM of healthy tissue and tumors is different. The tumor has a more dense and stiff ECM compared to healthy tissue, due to the formation of fibrous connective tissue[24,28]. This stiff and dense ECM creates a physical barrier for drugs resulting in impaired diffusion of the drug through the tumor. Leading to a higher chance of therapeutic resistance. In addition, the tumor ECM also hampers the diffusion of nutrients and oxygen, which creates hypoxia regions in the tumor, thereby, further impairing the diffusion and therapeutic effciency[17,29].

The ECM is degraded by proteases, which enable the remodelling of the ECM, which is important for different processes such as angiogenesis and wound repair in healthy tissue[30]. However, in tumors the proteases play a key role in tumor cell migration, invasion and metastasis of cancer by degrading the ECM and thereby allowing these processes. Examples of proteases are caspases, matrix metalloproteinases, cathepsins and urokinases. Matrix metalloproteinases play an important role in connective tissue remodelling, since matrix metalloproteinases can degrade all parts of the ECM.

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc containing endopeptidases. More than 30 types of MMPs have been identified[31,32]. MMPs are upregulated in many types of cancer and in particular MMP-2,-3,-9 and -14 are associated with malignant tumors. MMP-2 and MMP-9 can degrade the collagen IV in the basement membrane, which is a thin layer of ECM on which endothelial and epithelial cells grow and which separates the epithelia and endothelia from the connective tissue. Degradation of the basement membrane is important to enable tumor cells to become invasive and metastasize[32,333].

Hypoxia

Hypoxic areas in the tumor are not only caused by impaired diffusion of oxygen to all areas of tumor, as a result of the stiff and dense ECM, but is also a result of the disorganized blood vessels in the tumor. Cancer is characterized by uncontrolled cell proliferation. This rapidly grow of the tumor cells generates a high demand of oxygen. To supply for this high demand, the tumor starts to create its own microvascular network via angiogenesis. However, this tumor vasculature is chaotic and disorganized, which is in contract to the well-organized, hierarchically-branched vasculature system in healthy tissue. The unorganized vasculature in the tumor creates high variability in oxygen supply to different parts of tumor due to difficult diffusion through the tumor[34]. Approxiametely 50% of solid tumor cells receive insufficient oxygen. In particular the cell in the centre of the tumor, at a distance of 70 µm or further from the blood vessels, are oxygen deprived resulting in a hypoxic area in the tumor[35]. Hypoxia is presence in 60% of the solid tumor and is associated with poor therapeutic outcome since hypoxic

areas are difficult to reach for anti-cancer medicines[36]. The unreachable cells are not killed by the anti-cancer drug and might repopulate the tumor[34].

Acidic environment

This hypoxia state in tumor causes a metabolic shift. Cells start to switch from oxidative phosphorylation to glycolysis, which results in acidification of the tumor. During oxidative phosphorylation, glucose is converted into pyruvate, which produces carbon dioxide via the tricarboxylic acid cycle (TCA). However, during hypoxia, the TCA cycle is inhibited, while the glycolysis pathway is activated[37]. In this case, the produced pyruvate will not enter the TCA cycle, but instead will be converted into lactate via the glycolysis pathway[37,38].

Lactate is acid and high amount of lactate production leads to the acidification of the tumor microenvironment[39]. The pH in the environment of solid tumors is between 6.5-7.2, while the pH of healthy tissue is around 7.4[40].

Elevated levels of ROS and GSH

All types of cancer show also elevated levels of ROS. When the amount of ROS reaches a certain level, it can cause damage to the DNA and other cell structures. If the amount of ROS keeps increasing, it will eventually result in cell senescence or even cell death in normal physiological settings. However, when the circumstances are abnormal such as in cancer, high levels of ROS do not result in cell death. It can even be beneficial for tumor progression, since it promotes cell growth and angiogenesis[41,42]. The survival of cancer cells under those elevated levels of ROS can be explained by the simultaneous increase of glutathione (GSH).

GSH is an antioxidant and is important for the detoxification of different agents. The capturing of free ROS prevents cancer cell death under high amounts of ROS[42]. The expression of GSH is four times higher in tumors than in healthy tissue[43]. The GSH pathway is also responsible for the detoxification of several chemotherapeutic drugs by cleaving the disulphide bonds resulting in less available drug and thereby in impaired therapeutic efficiency[44].

All above described tumor characteristics, including low pH, hypoxia state, high MMP expression and elevated levels of GSH, could be used as internal stimulus for TME sensitive nanomedicines.

How can the tumor microenvironment be utilized to enhance the therapeutic efficiency of anti-cancer nanomedicines?

Charge-adaptable nanomedicines to enhance both systemic circulation, and cellular uptake

As described before, positively charged particles are more susceptible for opsonization, which marks them for elimination by phagocytes. To increase the systemic circulation, it would be preferred to use negatively or neutrally charged nanoparticles, which show longer circulation times[19]. However, multiple studies have shown that positively charged nanoparticles are better internalized by cells than neutrally of negatively charged nanoparticles due to the negative charge of the cell membrane[19,26]. There is even observed that positively charged nanoparticles have a 2.5-fold higher accumulation in tumor cells as a results of better tumor penetration and cellular uptake[19]. In conclusion, the perfect nanoparticle would be negatively or neutrally charged in the systemic circulation to prolong circulation time, and become positively charged in the TME to enhance tumor penetration and cellular update.

The change from negatively or neutrally charged to positively charged after tumor accumulation can be achieved by using TME-sensitive nanoparticles. These particles undergo a change in charge in presence of the specific characteristics of the TME, e.g. low pH or increased levels of MMPs.

The slightly lower pH in the TME compared to healthy tissue can be utilized to generate pH-sensitive nanomedicines. These nanomedicines undergo changes when entering the tumor area. One possibility is the use of polymers with ionizable groups, these groups become protonated or deprotonated at different pH levels resulting in a change of conformation or solubility of the polymer[40,45].

An ionizable is a group that is neutral but can become charged by accepting or releasing a proton. There are two types of ionizable groups, basic and acidic ones. Basic polymers can accept protons in an acidic environment (low pH), while acidic polymers release protons in a basic environment (high pH). Basic polymers are most interesting since the TME is slightly acidic and therefore useful in creating pH-sensitive nanomedicines. When the basic group accepts a proton, the group will change from neutrally to positively charged. Basic ionizable groups that are commonly used include amines, morpholines, pyridines and piperazines[45]. The most popular ones are amines because they are easy to prepare and their pKa is tuneable. In a study by Yang, J. *et al.*, an amine group was used as ionizable group to create a charge-reversable Doxil.

As mentioned before, Doxil was the first FDA approved nanomedicine against cancer, which is a DOX encapsulated in a PEGylated liposome. The PEGylation is important to increase the circulation time and causes less side effects to healthy cells. However, the PEG layer complicated the cellular uptake after accumulation in the tumor. The difficult cell internalization is partly caused by the slightly negative or neutral charge of the PEG, while a positive charge is preferred for cellular uptake.

Yang, J. *et al.* created a charge-reversal liposomal doxorubicin (CRDOXIL) to increase the cellular uptake by cancer cells, while maintaining the advantages of PEGylated liposome. The surface of CRDOXIL is negatively charged causing prolonged blood circulation and minimal uptake by healthy cells, thereby minimizing the cytotoxicity to the healthy cells[46].

The PEG component contains an acid-labile amide group, which becomes hydrolysed in the acidic environment of the tumor. Hydrolyse of the amide group results in release of the PEG and protonation of the amine ($–NH_3^+$) exposing a positive charge on the surface (Figure 2). The study showed that the CRDOXIL indeed resulted in higher cellular uptake compared to Doxil.

In addition, the antitumor activity against tumor cells and cytotoxicity to healthy cells was tested. It was observed that CRDOXIL resulted in low cytotoxicity to normal cells, which was comparable to Doxil. While the CRDOXIL also showed high anticancer activity, the performance was similar to free DOX. In conclusion, an acidity-induced charge-reversal DOXIL was successfully generated, which showed increased cellular uptake and demonstrated high anticancer activity, while cytotoxicity against healthy cells remained low[46].

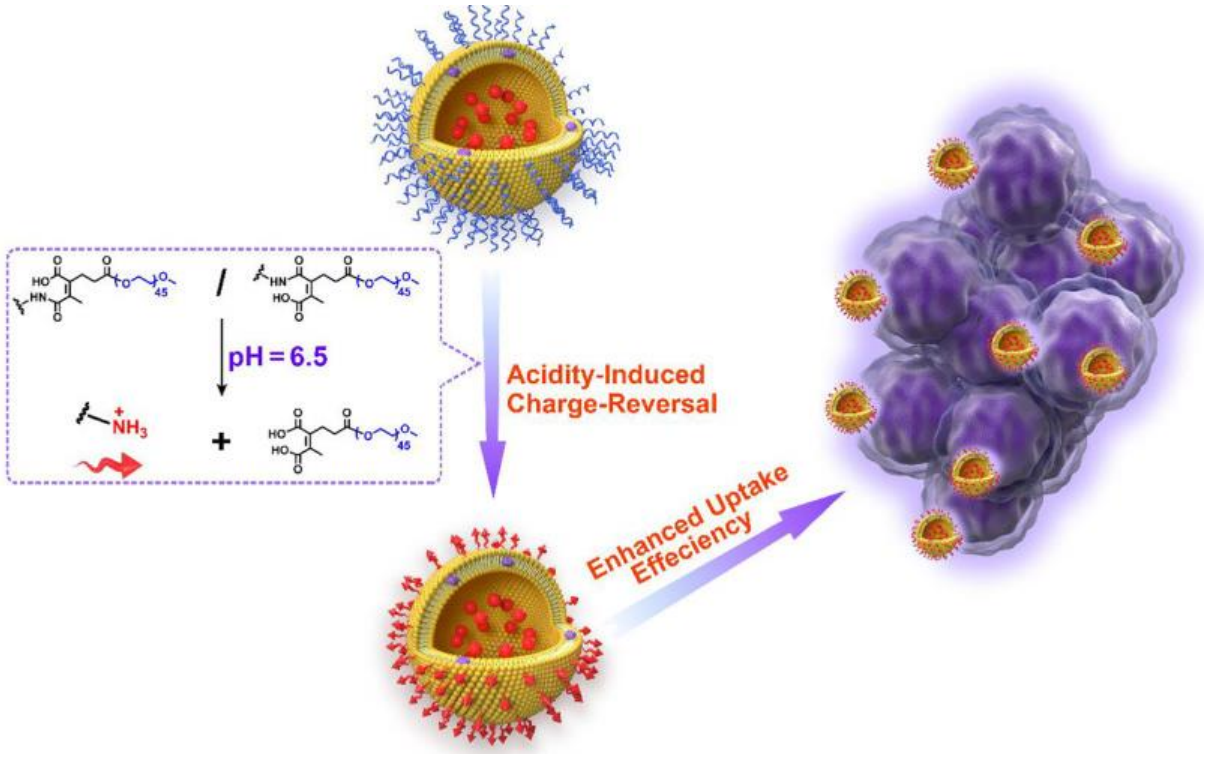

Figure 2:Charge-reversal liposomal doxorubicin. A DOX is encapsulated in a PEGylated liposome. At a pH of 6.5, the amine group is hydrolysed resulting in the release of PEG and the exposure of positive groups at the outside of the liposome. Thereby, enhancing the cellular uptake. Adapted figure[46]

Su, Z. *et al.* investigated a polymeric micelle composing of mPEG-C=N-PAsp(MEA)-CA copolymers (Figure 3). CA is the hydrophobic cholic acid (CA) core enabling transport of hydrophobic drugs, such as DOX. PEG is the hydrophilic outside and is important to increase the systemic circulation time. The main chain contains a pH sensitive benzoic imide bond between the PEG and polyaspartic acid (PAsp), which becomes hydrolysed in a slightly acidic environment, making it a suitable option as TME-sensitive linker[47].

In the study, it was shown that cleavage of the benzoic imine bond occurred at a pH of 6.5. When the benzoic imine bond was hydrolysed, the charge changed from slightly negative to positive (Figure 3). The cellular uptake was investigated at a pH of 7.4 and pH 6.5 and it was observed that there was a higher cellular uptake at pH 6.5 compared to pH 7.4 due to the positive charge at pH 6.5. These results showed that a pH of 6.5 is sufficient for cleaving the benzoic imine bond, subsequently resulting in a positive charge, which resulted in a more effective cellular uptake. An *In vitro* test assessed the anticancer effect at pH 6.5 and 7.4 and showed that an improved cell killing was observed at a pH of 6.5 due to the increased cellular uptake[47].

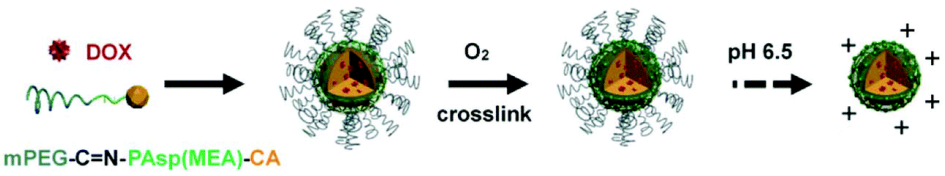

Figure 3: : pH-sensitive polymeric micelles. The polymeric micelles compose of mPEG-C=N-PAsp(MEA)-CA copolymers and are loaded with DOX. At a pH of 6.5, the benzoic imine bond between PEG and PAsp is hydrolysed, resulting in a change in charge from negative to positive which improves the cellular uptake. Adapted figure[47]

Instead of pH, Zhu, L. *et al.* used the elevated levels of MMP-2 to acquire a change in charge. They studied neutrally charged polymeric micelles, which become positively charged after MMP-2 cleavage.

The micelles consist of conjugates that self-assemble into the micelles. One conjugate includes PEG-pp-PEI-PE, where PEG is orientated to the outside and PE to the inside (Figure 4). PE is short for DOPE, which stands for 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine. PE is the lipid core of the micelle allowing transport of hydrophobic drugs. Polyethyleneimine (PEI) is a cationic polymer responsible for the positive charge of the inner layer of the micelle[48]. Polyethylene (PEG) is a layer protecting the micelle from rapid clearance in the circulation and gives a neutral charge to the micelle. PEG and PEI are connected by a MMP-2 sensitive peptide. The peptide will be cleaved in presence of MMP-2 resulting in the release of PEG, while the micellar structure of PEI-PE stays intact (Figure 4).

In the study was shown that incubated with MMP-2 indeed resulted in cleavage of the peptide, removing of the PEG layer and thereby exposing PEI at the outside. Moreover, they observed an increase of PEG-pp-PEI-PE inside the tumor cells compared to the uncleavable counterpart indicating that exposure of PEI on the outside improves the cellular update. They also investigated the adsorption of blood proteins on PEG-pp-PEI-PE and showed this adsorption was minimal. In conclusion, the study showed that the PEG layer probably would result in prolonged circulation time since the blood protein adsorption was minimal, while there is cellular uptake due to the exposure of PEI after cleavage by MMP-2[49].

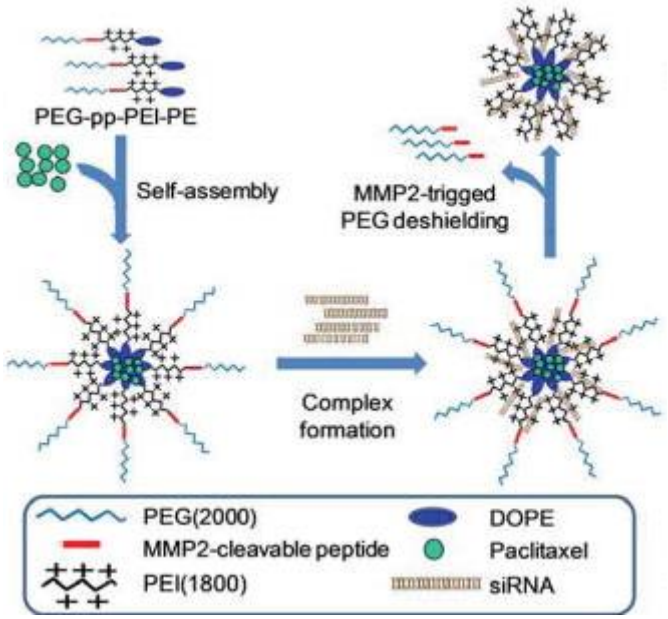

Figure 4: MMP-2-sensitive polymeric micelles. Self-assembly of PEG-pp-PEI-PE conjugates results in the forming of the micelle. The micelles are loaded with the drug Paclitaxel. In presence of the MMP-2, the bond between PEG and PEI is cleaved, resulting in the release of PEG. As a result, the positive charged PEI is exposed at the outside, giving a positive charge to the micelles[49].

Size changing nanomedicines to enhance tumor penetration

As mentioned before, nanomedicines with a size of 100-200 nm are preferred to prolong blood circulation time and to allow passive accumulation in the tumor via the EPR effect. However, a nanomedicine with a small size is favoured for deep tumor penetration, which is important to reach more distal areas of the tumor.

To meet both criteria, a nanomedicine should be created that can shrink in size after reaching the tumor. It allows both passive accumulation into the tumor and deep penetration in the tumor. Multiple

option of size changing nanomedicines are developed over the last years, including matrix metalloproteinase-sensitive size changing nanomedicines and pH sensitive shrinking nanomedicines.

Matrix metalloproteinase as trigger for shrinking of nanomedicines

There are multiple option to generate a MMP sensitive shrinkable nanomedicine, for example covering a MMP substrate with anti-cancer drugs or using a nanoparticle linked to an anti-cancer drug via a MMP sensitive bond.

In a study by Ruan, S. e*t al.,* a nanoparticles was created that reduced in size in response to matrix metalloproteinase. Gelatin nanoparticles were used and the nanoparticles were covered with gold-DOX-PEG particles (Figure 5). The complete structure has a size of 186.5 nm, which is favoured for prolonged circulation time and accumulation in the tumor via EPR[50].

After accumulation in the tumor, the gelatin nanoparticles covered with DOX are exposed to overexpressed metalloproteases, in particular MMP-2. MMP-2 is capable of efficiently hydrolysing gelatin[51], which results in shrinking of the nanoparticle. They showed that the size of the nanoparticle decreased from 186.5 nm to 59.3 nm in presence of MMP-2.

The penetration was investigated *in vitro* in tumor spheroid. Tumor spheroids normally show poor drug penetration and are therefore a suitable model to test the penetration. It was confirmed that the gelatin-gold-DOX-PEG had difficulties diffusing to the deep region of the tumor spheroid. However, after incubation with MMP-2 for 12 hours, the penetration was significantly improved, underlining the importance for small nanoparticle size for deep penetation[50].

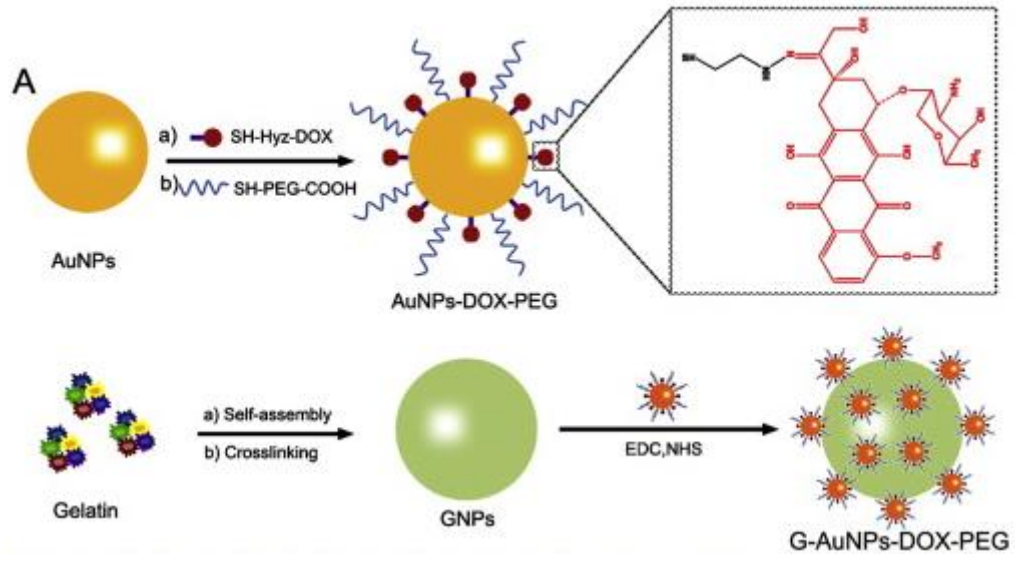

Figure 5: MMP-2 sensitive size shrinkable nanoparticle. Gold nanoparticles (AuNPs) linked to DOX and PEG are generated. The AuNPs-DOX-PEG nanoparticles are used to cover gelatin nanoparticles to create a MMP-2 sensitive size shrinkable nanoparticle[50].

The anti-tumor effect of the nanoparticle was investigated in 4T1 (breast cancer cell line) and B16F10 (melanoma cell line) tumor bearing mice. It was shown that treatment with gelatin-gold-DOX-PEG resulted in tumors with the smallest size compared to treatment with free DOX or gold-DOX-PEG without the gelatin part in both tumor bearing mice. Although, there are difference in the cancer type, the gelatin-gold-DOX-PEG was effective in reducing both tumor sizes[50].

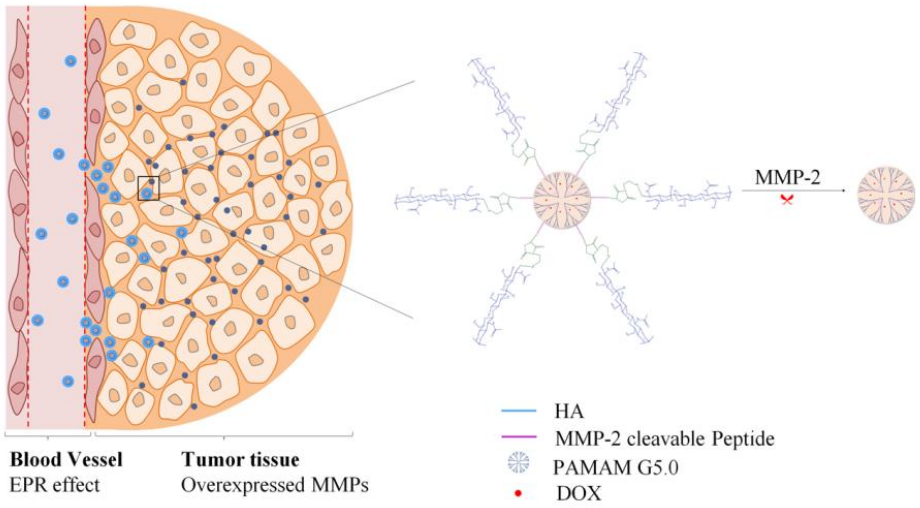

Figure 6: MMP-2 sensitive shrinkable PAMAM dendrimer. The PAMAM dendrimer is loaded with DOX and linked to HA via a MMP-2 cleavable peptide. The peptide is cleaved in presence in MMP-2 resulting in the removal of HA and thereby the reduction in size of the nanoparticle[22].

Han, M. *et al.* investigated a different type of MMP-2 sensitive anti-cancer nanomedicine (Figure 6). In this case a MMP-2 cleavable peptide was used, which would result in cleavage of the peptide in presence of MMP-2 and thereby shrinking of the particle.

They used a poly amidoamine (PAMAM) dendrimer loaded with DOX and linked to hyaluronic acid (HA) via a MMP-2 cleavable peptide. In presence of MMP-2, the peptide would be cleaved, thereby removing the HA outside resulting in PAMAM dendrimer containing DOX alone (Figure 6).

The size of the HA-pep-PAMAM nanoparticle was measured around 200 nm, which is advantageous for tumor accumulation. The PAMAM dendrimers alone are small particles and are therefore beneficial for deep tumor penetration. It was shown that after 4 hour incubation with MMP-2, the size significantly reduced from ~200 nm to ~10 nm. In tumor spheroids, it was showed that the penetration of HA-pep-PAMAM/DOX was increased after pre-treating with MMP-2, which confirmed that particle shrinkage resulted in deep penetration.

Beside deep penetration, the HA-pep-PAMAM showed also prolonged circulation time and tumor accumulation due to the size and the presence of HA. The large size is important for reduced clearance in the circulation and for accumulation in the tumor via the EPR effect. In addition, the tumor accumulation is further enhanced by HA, since HA has a high affinity for CD44, which is overexpressed on cancer cells.

The anti-tumor effect was investigated in tumor bearing mice. The tumor of the mice treated with HA-pep-PAMAM/DOX showed a higher tumor inhibition rate compared to DOX free and showed less side effects. In conclusion, HA-pep-PAMAM/DOX enhanced tumor penetration and thereby the anti-cancer effect, while having reduced side effects[22].

Besides metalloprotease, pH can also be used to generate size shrinkable nanoparticles. Li, J. *Et al.,* investigated pH sensitive shrinkable micelleplexes. The micelleplexe is a stable complex, which consists of triblock copolymer micelles and PAMAM dendrimers containing cisplatin prodrugs (Pt(IV) which form one complex through electrostatic interaction at pH 7.4[52].

The micelles have a PEG layer, giving the micelleplexes a hydrophobicity, which is important for long blood circulation. The complete micelleplexes have a size of 100 nm enabling passive accumulation in the tumor via the EPR effect[52].

The PAMAM dendrimers are positively charged, while the inner part of the micelle is negatively charged at pH 7.4. The charges allow for an electrostatic interaction between the PAMAM dendrimers and the micelles. However, in the acidic environment of the tumor, the electrostatic interaction is disrupted resulting in the release of the PAMAM dendrimers containing the cisplatin prodrugs. The PAMAM dendrimers have a small size and positive charge, which is both advantageous for deep tumor pentration[52].

Diffusion to hypoxic tumor regions to improve therapeutic efficiency

Size-shrinking nanoparticles can be used to improve the tumor penetration by creating a nanoparticle that is smaller than the mesh size of the ECM. Deep tumor penetration is also important to reach the hypoxic areas of the tumor. If tumor cells cannot be reached and killed by the anticancer drug, these cells can repopulate the tumor. Therefore, it is important to generate a nanoparticle that kills cells in the hypoxic area. This could be achieved by creating a nanoparticle which solely releases the drug after reaching the hypoxic area. This ensures that also the tumor cells in the hypoxia area are killed, which prevents the repopulation of the tumor.

Reductases

In the tumor, the high demand of oxygen in combination with the limited oxygen supply results in hypoxia. As a result of the hypoxic state, the reductive stress increases in the cells, which results in the overexpression of reductases such as nitroreductase, quinone reductase and azoreductase. Reductases catalyse a reduction reaction, whereby a hydrophobic group is converted into a hydrophilic one.

Li, Y. *et al.* created a liposome containing a substrate for nitroreductase, nitroimidazole derivate, in the phospholipid membrane of the liposome (Figure 7). The nitroreductase changes the nitroimidazole derivate into transient intermediate. Under normoxic conditions, the intermediate is oxidized back into the original nitroimidazole derivative. However, under hypoxic condition, the intermediate is further reduced resulting in a reduced nitroimidazole derivate, aminomidazole. This reduced variant contains a $–NH_2$ group instead of $–NO_2$, thereby changing the hydrophobicity from hydrophobic to hydrophilic. In case of the liposome, the change in hydrophobicity results in the destabilization of the phospholipid bilayer of the liposome leading to the release of the drug (Figure 7)[36].

In vitro treatment with hypoxia, showed transformation from nitroimidazole into aminomidazole and disappearance of liposomes due to the destabilization of the liposome. Although a small drug release was observed under normoxic conditions, the release increased extensively under hypoxic conditions. The response by the liposome under hypoxia was confirmed *in vivo.* In addition, the anti-tumor efficacy was tested by treating xenograft mice harbouring a ~50 mm tumor. After treatment with hypoxia responsive DOX-liposomes the tumors were smaller compared to tumors treated with free DOX or non-responsive DOX-liposome. In addition, prolonged survival of treated mice was observed after hypoxia responsive DOX-liposomes treatment. Altogether, treatment with hypoxia responsive DOX-liposomes improves the anticancer efficacy.

In conclusion, it is possible to create hypoxia responsive nanoparticles, which solely releases their drugs after entering the hypoxic area. The hypoxia responsive nanoparticle showed improved anticancer efficacy[36].

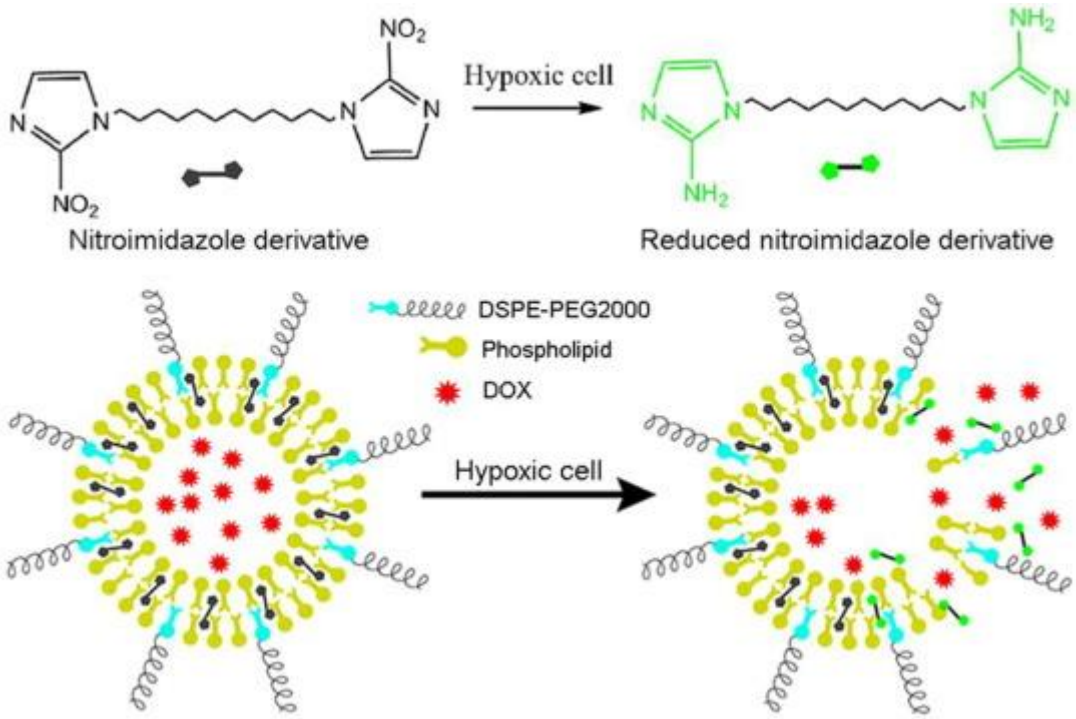

Figure 7: Liposome with hypoxia-triggered drug release. The liposome is loaded with drugs and stable under normoxic condition. When the liposome reaches a hypoxic cell, the nitroreductase causes a change in hydrophobicity of the liposome and thereby in release of the drug[36].

Disulfide bond as inner crosslink to reduce drug leakage in blood stream

Polymeric micelles are widely used as nanocarrier and were also used in the studies previously described. Despite their advantages, polymeric micelles also have a clear disadvantage, namely the chance for drug leakage in the circulation due to the dynamic characteristics of the micelles[47].

Polymeric micelles show poor stability *in vivo* resulting in reduced drugs accumulation at the tumor site and increased risk for systemic toxicity[53]. In addition, in some studies it was observed that pH-responsive nanocarriers showed drug release at a pH of 7.4, for example in a study by Zhang was shown that the anticancer drug PTX was released from 30% from the nanogels at pH 7.4 causing cytotoxicity to healthy cells[54].

To avoid drug leakage, nanocarriers can be further enhanced by adding a crosslink to the nanocarrier to improve stability. The most straight forward solution is to include a disulfide bond as crosslinker since disulfide is cleaved by GSH, which is also overexpressed in the TME and inside tumor cells. In this way, the crosslinker is only cleaved after reaching or entering the tumor, thereby preventing drug leakage in the systemic circulation.

Su, Z. *et al.* investigated a pH charge adaptable polymeric micelle, as described in 'Charge-adaptable nanomedicines to enhance both systemic circulation, and cellular uptake'. In addition, they included thiol groups in the side chain of the copolymer mPEG-C=N-PAsp(MEA)-CA to create disulfide crosslinks in the interlayer since thiol groups contain sulfide which can form disulfide bonds (Figure 8).

The micelles changed in surface charge after tumor accumulation, which showed improved cellular uptake. After the cellular uptake, the disulfide bond is cleaved as a result of the elevated levels of GSH inside the tumor cells, followed by the release of DOX inside the cells (Figure 8). Minimal drug release was observed without GSH indicating that the disulfide bond was efficient in preventing drug leakage.

In addition, a higher drug load content was observed for the cross-linked micelle compared to the non-crosslinked due to the capture of the drugs in the cross-linked interlayer.

In conclusion, the additional disulfide bond in the micelles is useful to prevent drug leakage and can even improve amount of drug load content[47].

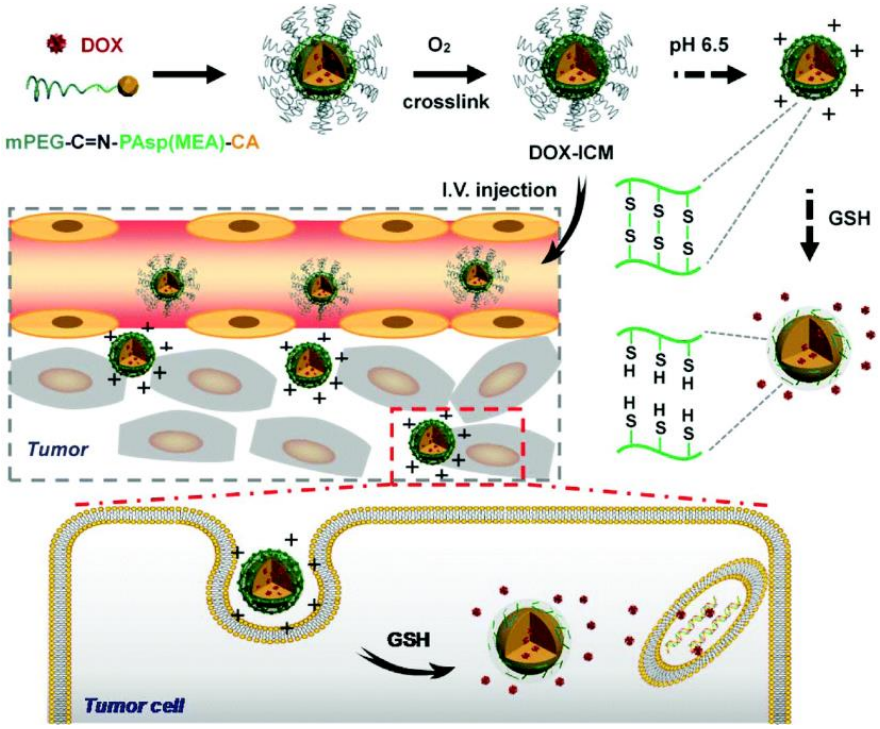

Figure 8: Dual-sensitive polymeric micelles. The polymeric micelles compose of mPEG-C=N-PAsp(MEA)-CA copolymers and are loaded with DOX. At a pH of 6.5, the benzoic imine bond between PEG and PAsp is hydrolysed, resulting in change in charge from negative to positive, which improves the cellular uptake. After cellular uptake, the micelle is exposed to GSH, which results in the cleavage of the disulfide bond in the micelles. Thereby, releasing the DOX from the micelles[47].

Disulfide bonds in nanomedicines to reverse chemo therapeutics resistance

GSH contributes to resistance development towards chemo therapeutics by cleaving the disulfide (SS) bond in chemotherapeutic drugs and thereby detoxifying it[44]. The therapeutic outcome of chemo therapy is impaired due to the elevated levels of GSH in the TME.

An example of a drug that shows drug resistance in tumor cells is cisplatin. Cisplatin is a chemotherapeutic drug and is used in the treatment of a broad range of tumor types[55]. However, tumor cells start to develop drug resistance against cisplatin, which impairs the therapeutic efficiency. A correlation is observed between cisplatin resistance and elevated levels of GSH. Therefore, Ling, X. *et al.* investigated the option to reduce the levels of GSH to improve the sensitivity towards cisplatin.

In their study, they generated a nanoparticle containing a high number of disulfide groups. The disulfide groups help to consume the available GSH, which leaves less available GSH for cleaving the drugs. Thereby, increasing the sensitivity towards cisplatin.

As a model, they used mice with ovarian xenograft tumors with resistance towards cisplatin. The effect on tumor growth was tested in these mice after treatment with free cisplatin or nanoparticles containing high number of disulfide groups loaded with drugs. The group treated with free cisplatin showed tumor growth, thereby confirming the resistance towards cisplatin. In contrast, inhibition of tumor growth was observed for the group treated with the nanomedicine. Afterwards, the tumors were collected and the tumors treated with nanomedicine showed markers of necrosis. In conclusion,

using a nanomedicine with high number of disulfide bonds improved the therapeutic outcome by GSH-scavenging to protect the drug from cleavage[56].

Although, the therapeutic outcome was investigated in the described study, nothing was mentioned about the effect on the levels of GSH in the tumor. Yang, *et al.* did asses the GSH concentration after using synthesized nanoparticles containing high number of disulfide bonds (known as DSNP). In addition, they also investigated the effect on the GSSG/2 GSH redox balance.

The thiol group (-SH) in GSH is exchanged with the disulfide bond resulting in the cleavage of the disulfide bond. During this reaction GSH is oxidized into oxidized glutathione (GSSG)(Figure 9)[57]. Cleaving disulfide bonds changes the redox balance towards GSSG. Changing the redox balance can be beneficial for enhancing apoptosis and reducing metastasis, thereby enhancing the drug efficacy and therapeutic outcome[58].

Yang, *et al.* synthesized nanoparticles with high number of disulfide bonds. In addition, they also created DSNP linked to all-trans retinoic acid (ARTA), which is used in the treatment of cancer. The effect on intracellular GSH levels, redox potential of GSSG/2 GSH, cell viability and cell migration capability was investigated for both nanoparticles[58].

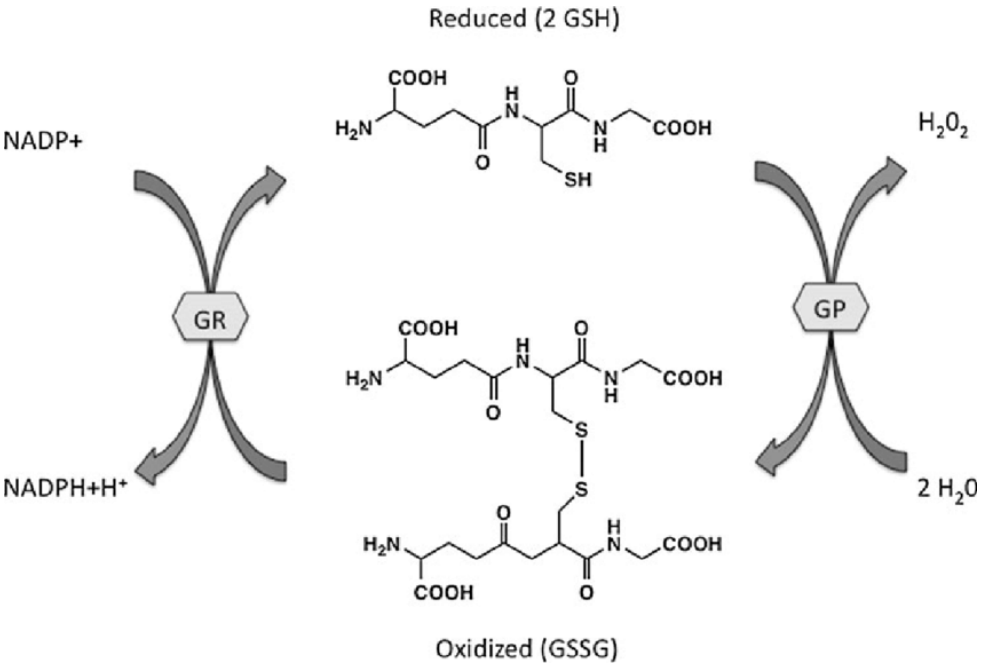

Figure 9: Redox couple GSSG and 2 GSH. In presence of reactive oxygen species, 2 reduced GSH are oxidized into GSSG[57].

After treatment with both DSNP and DSNP-ARTA, a significant decrease in intracellular GSH was observed in the used cell lines. Furthermore, also an increase in the redox potentials GSSG/2 GSH was measured for both treatments. In contrast, solely treatment with DSNP-ARTA resulted in decreased cell viability, showing that a combination of both, reduced levels of GSH and anti-cancer drugs, are essential to create drug-sensitive cells.

Finally, they investigated *in vitro* migration of cells by using a wound healing assay. If the cells can migrate, then they can effectively repair the wound. It was observed that the wound in untreated cells, DSNP or ATRA conditions was almost healed. In contrast, ATRA-DSNP showed only a 44% reduction in wound size instead of almost complete healing due to reduced migration of cells. Less cell migration is important in treating metastatic cancers.

In conclusion, combining nanoparticles with high number of disulfide bonds with anti-cancer drugs shows promising results for decreasing the intracellular levels of GSH and improving the therapeutic outcome, even for metastatic cancers[58].

Dual transformable nanoparticle

All previously discussed adjustments to the nanoparticles resulted in improved therapeutic outcome. If these adjusted could be combined, it might further enhance the therapeutic outcome.

In a study by Chen, J. *et al.* a nanoparticle was created, which combines change in charge, size shrinking and disulfide cross-link. This dual-transformable nanoparticle undergoes both size shrinking and charge changing as a result of the acidic environment in the tumor[59].

The shell-stacked nanoparticle (SNP) consist of a positively charged core, surrounded by a PEGylated and negatively charged shell (Figure 10). Shell and core are linked through electrostatic interaction. The core composes of polypeptides, which are cross-linked by disulfide bonds to maintain the small size of the core after shedding of the shell and prevent drug leakage.

The SNP has a size of 145 nm and is negatively charged, which characteristics are both advantageous for long circulation time. A DOX loaded SNP (SNP/DOX) showed an elimination half-life of 19.7 h, while free DOX showed a more rapid clearance of 11.4 h. In addition, the SNP/DOX resulted in a 1.8 timer higher accumulation in the tumor compared to free DOX.

When the SNP was incubated at a pH of 6.8, detachment of the shell occurred. As a result of this, the size reduced from 145 nm to 40 nm and the surface charge changed from negative to positive, both small size and positive charge enhance the penetration of a nanoparticle.

A non-transformable nanoparticle (NTNP) as control and the SNP were injected in a A549 lung carcinoma-xenografted mouse to compare the diffusing through the tumor. After injection, NTNP was mostly observed at the edges of the tumor, whereas SNP diffused through the tumor and could be measured closer to the centre of the tumor. It was observed that SNP could penetrate four times deeper than NTNP due to the small size of SNP compared to NTNP.

The positive charged surface enhances cellular uptake of the nanoparticle since the membrane of the cell is negatively charged. Inside the tumor cells, there are elevated levels of GSH, an enzyme responsible for the cleavage of disulfide bonds. The combination of high intracellular GSH levels and the disulfide bonds inside the core part of the nanoparticle results in accelerated drugs release inside the tumor cell (Figure 10).

In the end, tumor inhibition and DOX toxicity was tested *in vivo,* in A549 tumor-bearing mice. Mice treated with SNP/DOX showed a tumor inhibition of 97%, while free DOX resulted in a tumor inhibition of 75% and NTNP/DOX in 87%. Interestingly, SNP/DOX treated mice had no significant weight loss and showed no significant differences in biochemical parameters compared to healthy mice. Free DOX treated mice impacted the body weights of the mice drastically and could even result in dead. In conclusion, SNP/DOX demonstrated a higher tumor inhibition rate, with minimal toxicity compared to free DOX[59].

The study showed that it possible to create a nanoparticle that can change in charge and size after reaching the tumor and only releases the drug after reaching the tumor cells. This nanoparticle showed better penetration and tumor inhibition compared to a non-transformable nanoparticle.

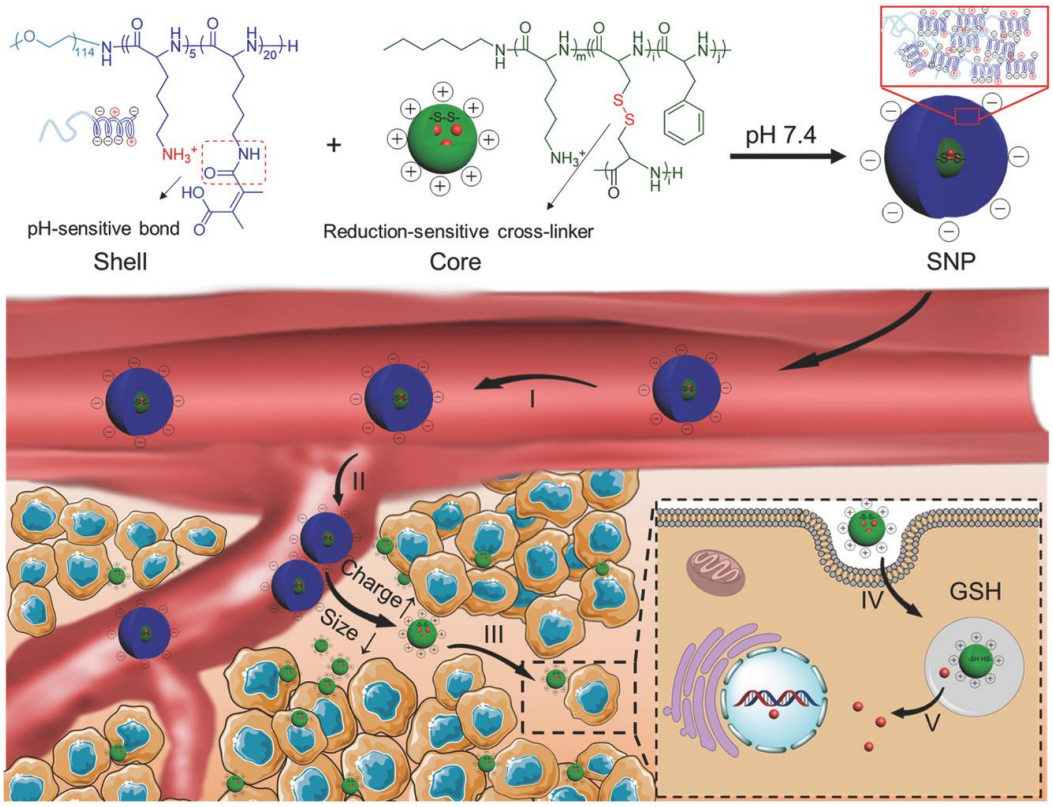

Figure 10: Dual transformable nanoparticle, loaded with DOX. The nanoparticle has a positively charged core, surrounded by a negatively charged shell. In the response to the slightly lower pH of the tumor, the nanoparticles changes in size and charge, which improves the cellular uptake. After cellular uptake, the disulfide bonds in the nanoparticle are cleaved by GSH resulting in the release of DOX[59].

Discussion

Nanoparticles are used to transport anti-cancer drugs through the circulation and deliver them at the tumor site. The use of nanoparticles improves tumor accumulation due to prolonged circulation time compared to free drugs. In addition, less accumulation in healthy cells should occur when a nanocarrier is used instead of free drugs[60]. The nanoparticles passively accumulate in the tumor via the EPR effect. Besides, the several advantages of nanoparticles, nanoparticles show limited therapeutic efficiency.

The low therapeutic efficiency is caused by the several challenges nanomedicines face during their route from administration till target site. These challenges include clearance from the blood circulation by the kidneys or mononuclear phagocyte system, accumulation at the tumor site, deep tumor penetration to reach distal and hypoxic areas and cellular uptake. In addition, drug release from the nanomedicines should only occur in the tumor.

To overcome these challenges, TME-sensitive nanomedicines were designed to improve therapeutic efficacy. These nanomedicines have a bond or group which are sensitive to a specific characteristics of the TME, e.g. low pH or overexpression of certain enzymes. In presence of the TME, the TME-sensitive group will change resulting in the controlled release of drug, charge conversion from negative to positive or size shrinking.

Nanoparticles were generated that change from a slightly negative charged nanoparticle into positively charged after accumulation in the tumor. The negative charge is important for long circulation time, while the positive charge improves the cellular uptake, both important to enhance the therapeutic outcome.

In addition, TME-sensitive nanoparticles were discussed that shrunk in response to the TME. The bigger size of the nanoparticle is crucial for long circulation time and accumulation in the tumor via the EPR effect. While, the small nanoparticles size is necessary for deep tumor penetration. Both could be achieved by this size shrinkable nanoparticle.

To ensure killing of tumor cells in the hypoxic area of the tumor, a hypoxia sensitive nanoparticles was designed. These nanoparticles would solely release their cargo after reaching a hypoxic area. Thereby, killing all tumor cells and preventing repopulation of the tumor.

At last, it was shown that disulfide bonds can be used to prevent drug leakage from the nanoparticles, thereby decreasing the cytotoxicity to healthy tissue and increasing accumulation in the tumor. In addition, the disulfide bonds contributed to lowering the GSH level, which is beneficial for overcoming chemotherapeutic resistance caused by cleavage of the SS bond in drug by GSH.

Although, it was shown that it was possible to enhance the therapeutic efficiency of an anti-cancer nanomedicine by making smart use of the TME, using the TME as a stimuli also has some drawbacks.

Firstly, the expression of all aspects of the TME can be highly variable between cancer types and between patients with the same cancer type. The levels of the TME stimuli might not be high enough to exceed the threshold of the TME-sensitive nanoparticle. Hence, the TME stimuli will not trigger a release of conformation change of the nanoparticle.

For example, the described pH-sensitive nanomedicines were tested at a pH of 6.8. However, the pH in the TME can vary between 6.4 and 7[61]. When the pH is above 6.8, the pH might be too high to induce changes in the pH-sensitive nanomedicines.

In addition, the pH-sensitive nanomedicines were generated to be sensitive to subtle changes in pH since the pH difference between healthy and tumor tissue is minimal. However, this makes the nanomedicines also more sensitive to intra and inter tumor pH differences.

Therefore, the heterogeneity between cancer types and patients might result in varying response to the nanomedicines and makes the response unpredictable[48,62,63].

Secondly, there is limited expression of characteristics of TME at early stage of cancer. These stimuli become only upregulated after substantial tumor growth. Therefore, the application in early stages tumors would be minimal[36].

Altogether, the expression of the aspects of the TME might highly vary between patients, tumor types and tumor stage. Therefore, an one size fits all might not be preferable and a more personalized nanomedicines could be more desirable.

One option would be to pre-screen the patient or use available information of patient. The patients can be divided into groups based on their genetic profile, comorbidities or exposure to certain environment compounds. The nanomedicine would be designed for the different patient groups, which would hopefully results in a more uniform response to the treatment[64]. In addition, biomarkers could be identified to decide which nanoparticle would be most suitable[65].

Another option to gain more information about the possible response in a patient would be by using a theranostics, which a combination between therapy and diagnostic. A theranostics contains both an image agent and an anti-cancer drug, which makes it possible to visualize the accumulation and the distribution of the drug through the tumor[2,66].

In a study by Fu, L. *et al.* a micelle theranostics was designed. The hydrophobic end of the unimers of the micelles was linked to either a fluorescent dye Cy5.5 or a sequencer and Dox was loaded in the core of the micelle. When the micelle was assembled, the Cy5.5 and sequencer were in close contact resulting in low fluorescence as a result of Fluorescence Resonance Energy Transfer (FRET). The micelle would dissociate under low pH condition, which would result in the release of Dox and a fluorescence signal since the distance between Cy5.5 and the quenched is too far for FRET[67]. The fluorescence signal would be a representation of the release of Dox in the tumor. However, the fluorescence probe was not attached to the drug. Therefore, early leakage of Dox in the circulation system would not be observed.

Besides TME, the EPR effect is also highly variable between patients and different tumor types. Most of the discussed TME-sensitive nanoparticles accumulate in the tumor due to the passive targeting mechanism of the EPR effect. However, if the EPR effect is highly variable, the accumulation of the nanomedicine in tumor is also highly variable. Thereby, making it difficult to predict the therapeutic outcome and impairing the therapeutic outcome for patient with a low EPR effect[68]. This could be overcome by adding a tumor targeting component to the nanomedicines, such as the previously discussed HA. By adding a targeting component, the nanoparticles will actively accumulate in the tumor based on tumor specific targets. The nanoparticle will not rely on the passive targeting mechanism of the EPR effect and can accumulate in the tumor independently of the highly variable EPR effect.

Although, the TME-sensitive changing nanomedicines showed promising results *in vitro a*nd *in vivo*, it is difficult to translate nanomedicines into the clinic due to several challenges. For commercial feasibility, a product should result in improved patient benefit such as less toxicity, enhanced therapeutic efficacy or less frequent administration to the patient. However, for nanomedicines it is

difficult to predict the therapeutic outcome since all data is based on animal study modes. The therapeutic outcome depends on tissue distribution, tumor accumulation, tumor penetration and drug release, which is highly different in animal study models than human patients. These differences make it more difficult to predict the clinical therapeutic outcome based on the observed efficiency in animal models[65]. Thereby, making it difficult to translate the nanoparticles to the clinic.

Secondly, production of the nanomedicines should be robust in terms of particle size, loading or encapsulation of drugs, and morphology[65]. Since some nanoparticles are generated via self-assembly, there is high variability between nanoparticles. This makes it difficult to have homogeneity within a batch of nanoparticles and homogeneity between batches. Not having a homogenous batch, makes it even more difficult to predict the therapeutic outcome, since for example the drug loading efficiency can be different every time. A certain dose can be beneficial for a patient when using a certain batch of nanoparticles, but might be inefficient or even toxic to the patient when using another batch. Hence, making it difficult to determine the optimal dose of the nanoparticles for the best patient outcome. Due to the low reproducibility of the nanoparticles, it is extremely difficult to translate it into the clinic[69].

Lastly, it is difficult to scale-up the production of the nanomedicines. The production of most nanomedicines is a complex process[65]. A complex production process makes it difficult to automate the production, which is import for upscaling the production. Without high production throughput, only a small number of patient can be treated, which reduces the change to have a commercial viable product.

A nanomedicine that would be able to overcome all challenges that impair therapeutic outcome, would shrink in size, change from neutral/ negative charge to positive charge, and contains disulfide links. Therefore, a dual transformable nanomedicine seems the best approach. However, every component that is added to the nanomedicine, adds an extra complexity to the production process, which makes it even harder to produce the nanoparticle and makes it more difficult to scale up the production.

In conclusion, different studies showed that it was possible to enhance the therapeutic efficiency of an anti-cancer nanomedicine by making smart use of the TME. However, there are still multiple challenges to need to be overcome before the TME-sensitive nanomedicines can make their entry into the clinic.

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