Ulm University

Department of Neurology

Director: Prof. Dr. med. Albert C. Ludolph

In vitro characterization of Nanoparticles engineered for brain targeted Curcumin delivery as treatment strategy for amyloid induced pathology in Alzheimer's disease

A dissertation submitted in partial fulfillment of the requirements for the degree "Doktor der Medizin" of the Medical Faculty of Ulm University

Johannes Robin Keller

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Current Dean: Prof. Dr. T. Wirth

First supervisor: Prof. Dr. A. Grabrucker

Second supervisor: Prof. Dr. B. Knöll

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List of abbreviations

%	percentage
°C	degree Celsius
µg	microgram(s)
µl	microliter(s)
µM	micromolar
Ab	Antibody
AD	Alzheimer's disease
AGE	Advanced Glycation Endproduct
APP	Amyloid precursor protein
APS	Ammonium persulfate
Aβ	Amyloid beta
BBB	Blood-brain barrier
CNS	Central nervous system
Cur	Curcumin
d	day(s)
DAPI	4,6-di-amino-2-phenylindol
ddH_2O	double distilled water
DIV	days in vitro
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid

ESCA	Electron Spectroscopy for Chemical Analysis
FCS	Fetal calf serum
Fig.	Figure
g7	glyco-heptapeptide
h	hour(s)
HBSS	Hanks' Balanced Salt Solution
HPLC	High performance liquid chromatography
i.p.	intraperitoneal
i.v.	intravenous
kDa	Kilodalton
M	molar (mole per liter)
MAP2	Microtubule associated protein 2
Mg	milligram(s)
Min	minutes
Ml	milliliter(s)
MRI	Magnetic Resonance Imaging
ms	Mouse
NFκB	Nuclear factor kappa-B
NP	Nanoparticle
PBS	Phosphate-buffered saline
PEG	Polyethylene glycol

PFA	Paraformaldehyde
PGA	Polyglycolic acid
PI	Protease inhibitor
PLA	Polylactide
PLGA	Poly(lactide-co-glycolic-acid)
PLL	Poly-L-lysin
Rb	rabbit
RCF	Relative Centrifugal Force
RES	Reticular Endothelial System
RNA	Ribonucleic acid
ROS	reactive oxygen species
Rpm	revolutions per minute
RT	room temperature
SDS	Sodium dodecyl sulfate
TBST	Tris-buffered saline with Tween 20
TEMED	N,N,N',N'-tetraacetylethylenediamine
Tris	Tris-hydroxymethyl aminomethane
V	Volt
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight

1. Introduction

1.1. Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disorder. It is the most common cause of dementia, with an estimated prevalence of 1.5 million in Germany in 2012 and 5.4 million in the USA in 2011 (Bickel 2014), (Ferri et al. 2005).

In 2015 an estimated 46.8 million people worldwide were suffering from some form of dementia. If the options for treatment and prevention remain the same, this number will double every twenty years with the current demographic changes. Worldwide, about 818 billion dollars were spent on dementia care in 2015 (Prince et al. 2015).

German Psychiatrist and Neurologist Alois Alzheimer first described AD in 1906 in his paper about certain peculiar diseases of old age. Unfortunately, now over one century later, there is still no specific diagnosis and no causal therapy for AD.

Clinically, AD displays an anterograde amnesia, which describes a difficulty making new memories. Changes in personality and temper have been reported. Furthermore, a reduction of semantic knowledge often emerges. The hippocampal area and the frontotemporal cortical areas are known to be affected the most. This explains the impact on learning, knowledge and personality. Additionally, almost any other area can be affected leading to a wide range of possible symptoms. There is literature about psychotic or depressive symptoms as well as sexual or aggressive behavior. Some patients tend to show childlike behavioral patterns (Butters et al. 1987).

Early stages of AD are usually nonspecific and can take decades to progress. The diagnosis can be challenging, since some of the early symptoms are often interpreted as normal aging processes. There can be issues with short term memory or fluency of written and spoken language. Apraxia, which is a difficulty to plan and coordinate movements, can be an early symptom, as well. These early sings are not specific enough to enable a certain diagnosis emphasizing the need for a novel diagnostic tool.

In moderate AD stages, there is a progression of the symptoms shown before. At this stage many patients are no longer able to cope with everyday life independently. Speech and motor skills decline, and psychiatric problems can intensify. Many patients become illusionary and lose the ability to fully grasp the meaning of their surroundings. Their reactions can sometimes vary from fear to aggression or general emotional lability. Relatives often report the neuropsychiatric changes to be much more challenging then the loss of knowledge or abilities.

Patients in advanced stages of AD will often be completely dependent on the help of caregivers. The progressive decline can result in the loss of speech and the inability to perform even simple tasks. In some cases, the agitation of moderate stages can change into complete apathy. Death is most likely to be caused by external factors such as infections or ulcers (Förstl H. et al. 1999).

There are at least three genetic predispositions for the early onset form of AD that affects patients under the age of 65 years. Mutations concern the genes of the Amyloid precursor protein (APP), the Presenilin 1 (PS1) and the Presinilin 2 (PS2). For the late onset form of AD which is defined to be perceivable after the age of 65 years, the Apolipoprotein E gene is known to carry variants increasing the risk of AD development (Pastor and Goate 2004).

While the diagnosis is based on clinical appearance, there are specific changes in the brain's anatomy that can be detected post-mortem. If they are severe they can also be seen in an MRI scan. The changes include a cerebral cortical atrophy affecting the frontotemporal brain most aggressively. The atrophy becomes apparent by a reduction of overall brain weight, a sulcal widening and a gyral narrowing. Primary motor, sensory, and visual areas are excluded while the frontotemporal cortex and hippocampus experience a significant shrinkage in the course of the disease (Scahill et al. 2002).

Different studies showed presymptomatic pathological changes in individuals that showed AD-like symptoms years later. This implicates that the disease begins long before it starts compromising the patient. Presymptomatic hippocampal atrophy can be detectable in individuals with familial AD (Fox et al. 1996).

1.2. Alzheimer's disease pathology

The Amyloid precursor protein (APP) is a transmembrane polypeptide with a length of 770 amino acids. In its physiological form it is known for neurotrophic activity. The APP in healthy phenotypes is cut into non-aggregating peptides by the α- and γ-secretase. The genetic predisposition and the environmental factors causing AD, allow a different cutting of the APP by the β- and γ-secretase (Vassar et al. 1999). The incorrect processing of the APP results in the appearance of the Amyloid beta peptide. In AD there is a pathological aggregation of this 42 amino acid long Amyloid beta peptide (Aβ 1-42) of about 4 kDa. The Aβ monomers form dimers, oligomers, protofibrils, and mature fibrils in that order. This leads to the formation of mainly extracellular Aβ aggregates. Aβ modifications like phosphorylation have been shown to increase aggregation and toxicity (Kumar and Walter 2011).

Amyloid plaques are big and inhomogeneous aggregates of the Aβ peptide and other molecules. AGEs (Advanced Glycation Endproducts) are built by non-enzymatic glycation. The plaque, being a pathological aggregate, causes microglial activation and neuroinflammation. Oxidative stress is induced that damages the surrounding neurons. Synaptic loss is well described and should be contributed to a combination of the toxic effects and the displacement of synapses by the plaques. Synaptic loss as well as a reduction in neuronal cell count both strongly correlate with the cognitive impairment in AD (Butterfield 1997). It has been shown that the main reason for cell loss in AD is the inflammatory response that is triggered by the Aβ plaques (Rogers, Webster, & Lue, 1996).

Extracellular plaques result in microglial activation and initiation of the NFκB signal pathway (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells). This causes an increase in proinflammatory mediators such as cytokines, chemokines, prostaglandins, leukotrienes, C-reactive protein and others. Under physiological conditions this reaction is needed to commence the immune response to fight pathogens and to clear cell debris. However, in AD with the plaques being persistent, a chronic inflammation occurs (Rogers, Webster, and Lue 1996). Microglial activation results in the release of reactive oxygen species (ROS) (Soman 2013).

Reactive oxygen species are usually supposed to neutralize pathogens like bacteria or parasites. They are highly reactive radicals like the hydroxyl radical, superoxide, peroxides and singlet oxygen. Unfortunately, they also damage healthy human cells by oxidation of lipids and amino acids and by direct DNA damage. This is the main reason for neuronal cell loss in AD (Butterfield 1997). There is some evidence that oxidative stress in AD may be especially severe in the early stages and decline with the progression of the disease (Nunomura et al. 2001).

The hyperphosphorylation and aggregation of intracellular tau protein constitutes the second toxic protein deposition in AD – the neurofibrillary tangles. It is believed to be caused by an imbalance of tau kinases and phosphatases. Phosphorylated tau loses its function as a microtubular protein and therefore more tau is synthesized. Increasing amounts of phosphorylated tau result in a loss of neuronal function and in progressive degeneration (Iqbal et al. 2005).

Synapse loss in AD is especially severe in hippocampus and neocortex and is also strongly correlated to clinical impairment. It has been shown that Aβ oligomers increase the probability of LTD – Long time depression by a calcineurin dependent pathway. This shift towards LTD results in spine shrinkage and ultimately a reduction of synapse density. Synapse count is a good cellular correlate to evaluate the cognitive impairment in AD. It has been shown that Aβ causes synapse loss in hippocampal cells (Shankar et al. 2007).

Different synaptic proteins are impaired in AD. In the early stages of the disease, there is a significant reduction of synaptophysin in the frontal cortex. This development can even start before the formation of visible plaques. During this time the amount of synaptotagmin and Growth Associated Protein 43 (GAP43) seem to increase as a compensatory mechanism. In advanced stages however, the amount of these two proteins and many more such as synaptopodin and Ras-related protein (Rab-3A) are decreasing (Masliah et al. 2001), (Reddy et al. 2005), (Barbara et al., 2017).

1.3. Curcumin

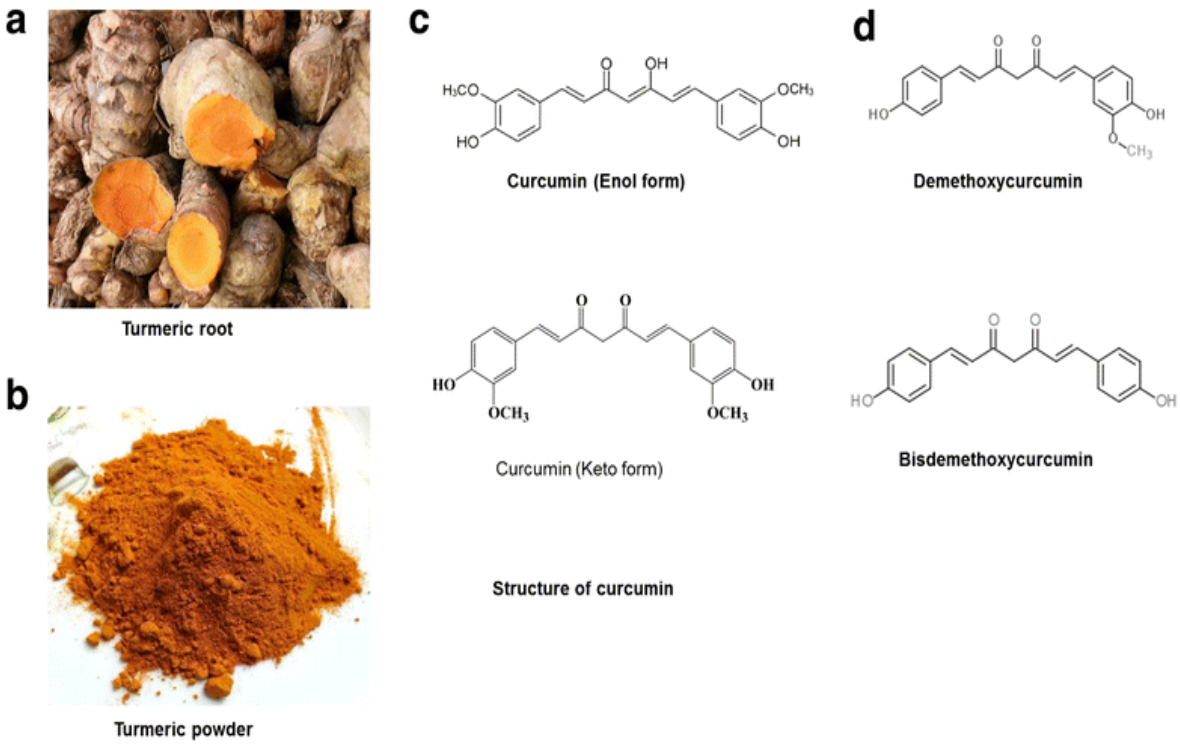


Figure 1: Source and conformations of Curcuminoids. a) The Tumeric root as natural source of Curcuminoids. b) Tumeric powder used as spice and drug. c) The structural formula of the enol and keto form of Curcumin. d) Demethoxycurcumin and Bisdemethoxycurcumin (Banik et al. 2017). https://doi.org/10.1186/s13046-017-0566-5 (open access), CC BY 4.0, https://creativecommons.org/licenses/by/4.0/

The diarylheptanoid Curcumin has been identified to be the main Curcuminoid and is used for the evaluations in this study. It can be found in the rhizome of the Turmeric / Curcuma longa plant, which contains about 3-5% Curcuminoids. 50-60% of these are Curcumin in its main conformation (1,7-Bis-(4-hydroxy-3-methoxyphenyl)-hepta-1,6-dien-3,5-dion). A keto-enol tautomerism is responsible for the different conformations. It has been shown that both conformations are able to bind to Aβ (Amyloid beta), while the enol form has the stronger effects. The enol form is more dominant in an alkaline medium while the keto form prevails in acidic mediums (Yanagisawa et al. 2010). The recommended Curcumin concentration is stated to be around 10 µM (Goozee et al., 2016).

Curcuminoids are natural polyphenols of orange-yellow color, and they display some of the typical effects phenols are known for such as antioxidative and anticarcinogenic properties. Many promising physiological effects have been described for Curcumin in recent years, including:

- Antioxidant effects: Radicals can be neutralized by two phenolic groups. Phenols which are also responsible for the health benefits of green tea or reasonable amounts of red wine are radical scavengers due to their ability to absorb singled electrons. Neutralized ROS can no longer oxidize lipid membranes, peptides or DNA. Curcumin has also been shown to activate Nrf2 (Nuclear factor 2) which leads to a greater expression of the antioxidant responsive element (ARE) (Esatbeyoglu et al. 2012).

- Anti-inflammation: Inhibition of NFκB (nuclear factor 'kappa-light-chain-enhancer' of activated B-cells) is achieved by the inhibition of the phosphorylation of IκB (Inhibitor of κB). Non-phosphorylated IκB will not be degraded and continues to inhibit the inflammatory effects of NFκB. This results in decreased levels of proinflammatory mediators like TNFα (Tumor necrosis factor α) or IL1β (Interleukin 1 β) (Lin et al. 1997). Overexpression of NFκB is involved in multiple pathologies such as chronic inflammatory or autoimmune diseases and many types of cancer. It also plays a role in different chronic diseases such as diabetes, arthritis and COPD (chronic obstructive pulmonary disorder) (Smahi et al. 2002). Immunocytochemical staining for NFκB in post-mortem brain slices showed significantly more NFκB expression in AD patients compared to healthy controls (Terai, Matsuo, & McGeer, 1996). This suggests that Curcumin and its related molecules could be suitable for the prevention and treatment of many frequent populational diseases including AD.

- Anti-amyloidogenic: It has been shown that Curcumin and other Curcuminoids are able to bind to the Aβ peptide. The two phenolic groups on each end of the Curcumin fit into the polar pocket of the Aβ. By doing so the aggregation is inhibited (Reinke and Gestwicki 2007). By binding to the aggregation site, Curcumin can not only inhibit the formation of new Aβ aggregates, but also disaggregates the ones that have built already (Yang et al. 2005). Curcumin has been shown to label senile plaques *in vitro* and *vivo* (Garcia-Alloza et al. 2007). The chelation of metal ions by Curcumin has been shown to decrease the rate of Aβ aggregation (Baum and Ng 2004).

- There are some epidemiological studies suggesting that there is a lower incidence and prevalence of AD on the Indian subcontinent where the Curcuma root is used as traditional spice and drug (Chandra et al. 2001). Traditional Indian and Chinese medicine has used Curcuma for ulcers, joint pain and a whole variety of internal diseases for thousands of years.

- Tau suppression: Neurofibrillary tangles consisting of phosphorylated tau aggregates are the second pathological build up in AD. It was proposed that Curcumin suppresses tau aggregation and induces its clearance by molecular chaperones (Ma et al. 2013).

- Anti-cancerogenic: The activation of tumor suppressor gene p53 by Curcumin has been shown to result in an increase of pro-apoptotic mediators like Bax in tumor cells (Lai et al. 2011). NFκB suppression has also been linked to positive effects in cancer treatment (Lin et al. 1997).

- Antibacterial/Antiviral: Beneficial effects of Curcumin against both gram negative and gram positive bacteria and against some DNA and RNA viruses have been published (Singh et al. 2010).

- Curcumin increases neurite formation and overall cell viability of neurons (Ray and Lahiri 2009). The improvement of overall cell survival seems to be the result of the effects listed above and possibly others not yet detected. For this study the free Curcumin was diluted in DMSO which is most likely to be accounted for the toxic effects at higher doses. It has been shown that DMSO has toxic effects on retinal cells *in vitro* (Galvao et al. 2014).

- Unfortunately, Curcumin exhibits limited oral bioavailability and poor blood-brain barrier (BBB) crossing. Therefore, the sheer oral consumption of Curcuma is not likely to result in an effective dosage at the site of the AD pathology. Loading the substance into NPs for i.v. or i.p. injection has been shown to increase the concentration and half-life of Curcumin within mice (Cheng et al. 2012). NPs with targeted release can be designed to enrich within the desired compartment. Specific ligands are used to initiate BBB crossing increasing the NP concentration in the central nervous system (CNS). Curcumin itself is known for Aβ affinity. This way Curcumin loaded NPs targeted for CNS drug administration can be used to increase the Curcumin concentration around the plaque while reducing the peripheral concentration.

1.4. Blood-Brain Barrier

The blood-brain barrier (BBB) is the physiological barrier limiting the exchange between the blood's circulatory system and the central nervous system (CNS). The endothelial cells of the microvessels in the CNS are surrounded by pericytes and covered by perivascular feet of astrocytes. The many tight junctions between the endothelial cells restrict the passage of substances across the BBB. The basal lamina surrounding the pericytes and the blood vessel is a non-cellular part of the BBB (Abbott, 2000).

The physiological functions of the BBB are of major importance for the maintaining of a normal brain environment. The ion homeostasis must be kept in a very narrow range, because small changes in the concentrations of potassium-, magnesium-, or calcium- ions would result in an altered cerebral excitability. The BBB also enables the CNS to regulate the concentrations of protein and glucose by using facilitated diffusion. Water is transported through channels, while messenger molecules and hormones are actively transported. This limitation of free diffusion allows the CNS to keep its chemical composition without being affected by peripheral changes. The neuroexcitatory amino acid glutamate is ingested after food consumption and could lead to severe overexcitation if it wasn't restricted by the BBB. Only a few very small or lipophilic molecules can cross freely. The BBB also helps to protect the CNS from circulating pathogens or toxins that could originate from some kind of wound or infection. This is important since the adult brain does not always have the ability to regenerate after being damaged (Abbott et al. 2010).

The size or chemical constitution of many known drugs is the reason why they cannot penetrate the BBB and therefore are not suitable for the treatment of neurological diseases. Many studies have been published concerning the application of therapeutic agents into the CNS. Nanotechnology is one of the most promising technological advances, because NPs can be loaded with a wide variety of substances.

Curcumin's limited permeability enables it to pass the BBB with only about 2% of the injected dose. It has been shown that loading the Curcumin into NPs will not only result in higher bioavailability in general but can also be used to target specific compartments.

Nanoparticles (NPs) conjugated with the g7 glycopeptide are actively taken up by the CNS increasing the Curcumin concentration within the brain to 15% of the injected dose (Tosi and Em 2011), (Tosi et al. 2007).

1.5. Nanoparticles

The IUPAC (International Union of Pure and Applied Chemistry) defines Nanoparticles as a "Particle of any shape with dimensions in the 1×10^{-9} and 1×10^{-7} m range". This definition contains particles from 1 – 100 nm, while particles from 100 – 2500 nm are defined as fine particles. In reality, particles up to 500 nm are accepted to carry the prefix "nano". NP size is important since particles bigger than 1 µm are taken up by the RES (Reticular Endothelial System) more rapidly which results in lower bioavailability of the loaded compound.

The small radius of a NP results in a very big surface area compared to the volume.

A sphere's surface area: $4\pi r^2$

A sphere's volume: $4/3(\pi r^3)$.

Ratio of surface area to volume:

$$\frac{4\pi r^2}{4/3(\pi r^3)} = \frac{3}{r}$$

This shows how a small radius results in a relatively big surface area compared to the volume of a sphere.

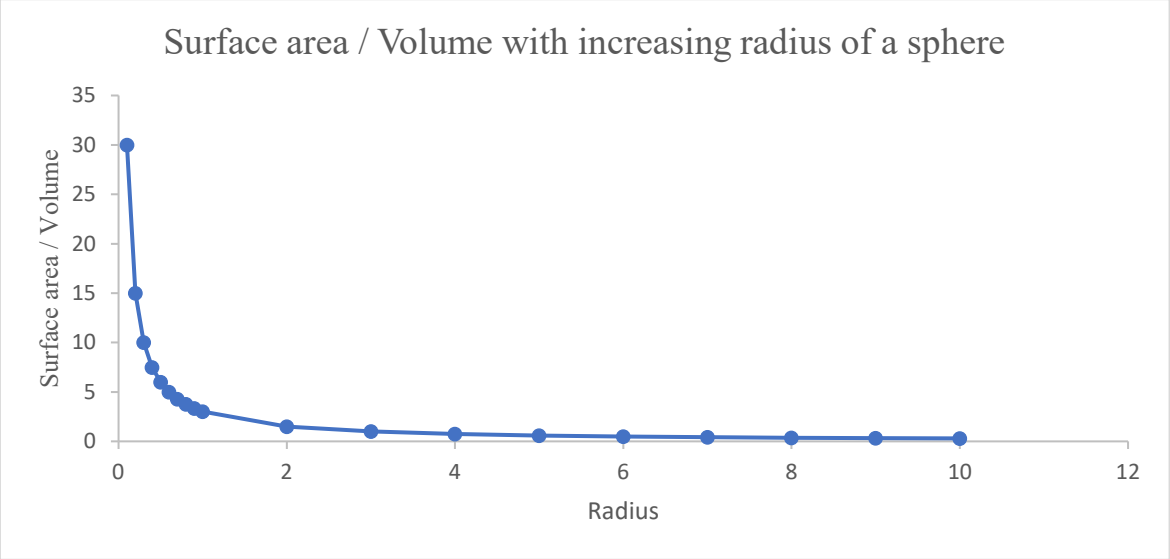


Figure 2: Visualization of the quotient of surface area and volume of a Nanoparticle with increasing radius.

With an increasing radius the volume of a sphere increases faster than its surface area. Dividing a big sphere into smaller spheres the volume will remain the same while the surface area increases. This visualizes that very small round particles have a bigger surface area in relation to their volume than bigger particles. This is advantageous for many possible applications because chemical reactions usually occur on the surface of a particle.

Another unique characteristic of NPs regards electromagnetic forces. While the gravitational forces between NPs are weak due to their small mass, the electromagnetic forces become more relevant.

There is a long list of possible applications for Nanoparticles as drug carriers. Studies have been performed in the field of vaccination, cancer, inflammation, cerebral diseases, pain management, regenerative medicine, cardiovascular or infectious diseases, osteoporosis, diabetes and more (Danhier et al. 2012).

The disaggregation of the Curcumin loaded NP in this thesis results in a sustained release of the Curcumin load. Optimal Curcumin loaded NP formulations could result in an effective Curcumin concentration in the CNS over a prolonged time frame after a single injection (Barbara et al., 2017).

1.6. Types of Nanoparticles

There is a wide variety of materials and production methods for NPs. A way to characterize them is by shape, size and chemical properties. These are the most common types of NPs listed in increasing size:

- Carbon nanotubes have a diameter of only a few nm while their length can greatly vary from the nanometer to the micrometer spectrum. They are made up by a single layer of carbon atoms shaped into a tube. There is great effort in research since they present unique electrical and mechanical properties. They are very resistant while being quite light. The possible technical applications are almost endless. In medical research, carbon nanotubes are tested as delivery systems.

- Quantum Dots (QDs) are fluorescent semiconductor nanocrystals of very small size. With a diameter of about 2-10 nm they often contain less than 100.000 atoms. The wavelength of the emitted light very much depends on the size and composition of the QD. Typical materials are semiconductors like silicon, zinc and cadmium combined with oxygen, nitrogen or sulfur. QDs are highly stable and can be used for *in vitro* and *in vivo* tracking. Conjugated with a tumor specific antibody they are tested for tumor diagnostics. (Gazouli et al. 2014)

- Dendrimers are highly branched polymers that can be divided into core, branches and surface.

- Liposomes and Micelles are both nanospheres of up to 100 nm. The only difference is their membrane, since Micelles are only composed of one layer while Liposomes have double lipid membranes. Both can be used to deliver a variety of substances.

- Metallic Nanoparticles are often smaller than 100 nm. The most common metals for their solid core are gold, silver and iron. Iron core Nanoparticles can be conjugated with surface ligands to bind to specific tissues. They do not only have diagnostic possibilities but can also be used for thermal ablation. *In vivo* iron core Nanoparticles can be heated from outside the body by an alternating magnetic field. This way they can be used in thermal tumor ablation therapy.

- Polymeric Nanoparticles come in all sizes and can be built out of diverse materials. Polymer in Greek means “out of many pieces”. These pieces are called monomers and bind to each other in a repetitive sequence. Polyethylene for example is a well-known polymer fabricated by the polymerization of Ethylene and is commonly used as plastic. Different monomers can be combined, as well. The PLGA poly(lactide-co-glycolic-acid) NPs used for this thesis are produced by the polymerization of lactic and glycolic acid. Polymers have some unique characteristics that make them especially suitable as a material for Nanospheres carrying a desired load. They can easily be modified and can be built to be biodegradable.

  (Nanoparticles Types, Classification, Characterization, Fabrication Methods, Springer 2016)

1.7. PLGA Nanoparticles

PLGA - poly(lactide-co-glycolic-acid) (PLGA-RG503) is a very promising polymer for Nanoparticle formulation. It is very well suited for drug delivery and tissue engineering. Lactic and glycolic acid can be polymerized in different ratios using sonication with the solvent evaporation method. For this study a PLA:PGA ratio of 50:50 was chosen which is known to have the shortest half-life of PLGA materials. This allows the production of polymeric NPs with a matrix of relatively weak polyester connections. This has many advantages:

1. PLGA Nanoparticles have a short half-life of only a few days or weeks depending on the environment and specific formulation. This alleviates the concerns about pathological accumulation within specific compartments like lungs, liver, spleen or kidney. The 50:50 ratio of lactic and glycolic acid displays the fastest degradation time. In other ratios the degradation time decreases with increasing amounts of glycolic acid (Vilella et al. 2014).

2. PLGA is considered safe, being used as a biodegradable polymer in surgical sutures for forty years (Ulery, Nair, and Laurencin 2011). It is approved by both the FDA – Federal Drug Administration and the EMA – European Medicines Agency.

3. By the time the NPs fall apart they release their load. The lactic and glycolic acids are further metabolized in the citric acid cycle (Krebs cycle) inside the mitochondria. This is the normal way of energy generation by oxidation in all eukaryotic cells, so the process is not likely to cause toxicity or hypersensitivity and can be performed by any cell in the human body.

4. It has been shown that PLGA Nanoparticles conjugated with the g7 peptide are able to penetrate the brain parenchyma with up to 15% of the injected dose in just two hours. This shows a significant enrichment, since the brain weight only accounts for 1-2% of the total body weight (Vergoni et al. 2009), (Tosi and Em 2011).

5. PLGA NPs can mask their load from opsonization and degradation by the immune system. This can most likely be attributed to the ability of PLGA NPs to attract peptides on their surface (Radic et al., 2015).

6. A large variety of drugs can be carried in PLGA NPs, like small molecules, proteins or nucleic acid. With the help of different formulation processes the loading of both hydrophobic or hydrophilic drugs is possible. NPs could enable the administration of many drugs that are not suitable for oral intake.

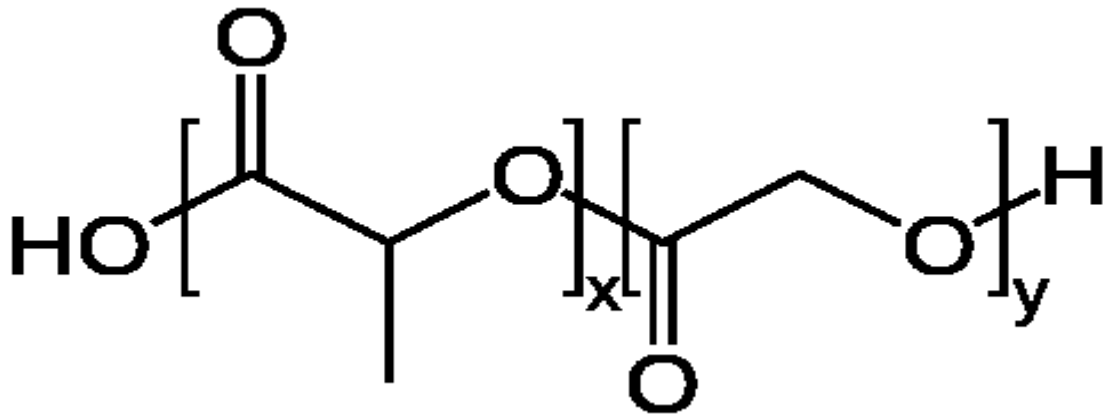


Figure 3: PLGA poly(lactide-co-glycolic-acid) polymerized from lactic (x) and glycolic (y) acid. (Fvasconcellos 2008) https://commons.wikimedia.org/wiki/File:PLGA.svg (21.01.2020) (public domain).

1.8. Nanoparticle modification

There are countless possible modifications for polymeric NPs. Ligands can be attached to the surface of the NP or can be included within the polymer matrix. These ligands can be modified even further, like the PEGylation of QDs in our case.

There can be multiple modifications simultaneously. NP modifications offer a variety of desirable possibilities but can also change the NPs physicochemical properties. Differences in size and surface potential can influence the toxicity and metabolism. For this reason every new NP modification has to be tested thoroughly.

1.8.1. G7 glycopeptide

g7: H_2N-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-Glucose)-$CONH_2$

The g7 glycopeptide is similar to endogenous opioids with only the last amino acid being altered. This way, a g7 coating can induce blood-brain barrier crossing without having the other effects of an opioid. Clathrin-dependent endocytosis is responsible for the active uptake of g7. Within the brain, it can induce further distribution via gap junctions and tunneling nanotubes (Vilella et al. 2014).

It has been shown that the g7 coating is able to increase the amount of Nanoparticles and their loaded drug in the brain in a significant manner (Tosi et al. 2007). G7 was mixed with the PLGA to make up 10% of the matrix.

1.8.2. Quantum Dots

Quantum Dots (QDs) are fluorescent semiconductor nanocrystals. Even though they are only a few nm in size they are very stable and resistant. Depending on their exact composition they can emit light of any visible wavelength. The strong fluorescence can be tracked both *in vitro* and *vivo*, enabling the observer to track NPs inside the living body (Gao et al. 2008) (Gazouli et al. 2014). The QD signal is quite bright and specific while there is only an average of a few QDs per Nanoparticle.

The 40 pmol of QDs used for the modification of 15 mg of NPs can show the exact location of the NPs while many other dyes cause a higher background signal. For the investigated NPs QDs from Molecular Probes® where used (Q21501MP). Light at a maximum of 608 nm is emitted.

PEGylation is the process of surface modification with polyethylene glycol (PEG). PEGylated QDs, like the ones used in this thesis express increased water solubility.

1.8.3. Rhodamine and Cyanine-5-amine dyes

For the NPs used in this study Rhodamine-B-piperazine was mixed with the PLGA in a ratio of 1:50. It is a fluorescent dye with an emission maximum of 560 nm when excited with 510 nm. The dye is used to detect the NPs *in vitro*. It is responsible for the red fluorescence of the NPs showing that they attach to the neurons surface. Rhodamine is not known to be toxic.

Cyanine-5-amine (Cy5) is an organic dye out of the polymethine group. It is contained in the PLGA matrix at a concentration of 5%. For this study we used Cy5 from Lumiprobe (1469277-96-0). It emits orange light of 710 nm when excited with 684 nm. There are no references of Cy5 to result in significant toxicity.

1.8.4. Targeting with Antibody coating

The covalent binding of monoclonal antibodies to the surface of NPs is a specific way of drug targeting. This can be quite effective since the NP's surface is enormous compared to its volume. In the case of this study NPs were coated with anti-Aβ antibodies binding to the amino acids 13-28. This way the NPs are more likely to localize close to plaques since they can bind the Aβ within them. By the time they break apart, the Curcumin load is at its target and there is less chance of degradation or cellular uptake in other tissues. This means higher concentrations at the site of pathology and less peripheral circulation and hence less side effects. The data of the antibody-coated NPs is not included, because the moderate antibody concentration of only 40 pmol per batch did no overcome the affinity that the Curcumin itself already has towards the Aβ.

In a similar approach it has been shown that PLGA NPs conjugated with NCAM1 or CD44 antibodies can selectively target neurons or glial cells. The NPs were used to deliver Zn^{2+} and could increase the intracellular concentration of the ion (Grabrucker, Garner, et al., 2011).

It has been shown that antibody coated NPs are able to entrap in the corresponding tissues. In the cited study NPs were used that are able to bind to MCF-7 in human invasive ductal breast carcinoma cells to enrich within the tumor (Kocbek et al. 2007).

1.8.5. Nanoparticle nomenclature

The matrix of all NPs in this study is made up of PLGA. However, there are differences regarding the ligands and the load.

In the name of the NP the load is stated after the "NP". The Curcumin loaded NP without any surface modifications is called NP-Cur.

Ligands are stated first, so an empty NP modified with the g7 peptide is called g7-NP. There can be multiple modifications simultaneously. The g7-Ab-NP-Cur has both g7 and antibodies on its surface while carrying the Curcumin load.

1.9. Theranostic Nanomedicine

The term theranostic refers to the combination of diagnostic and therapeutic functions within one application. This includes not only the visualization of drug delivery and release but also of the localization in the treated tissues. This can be realized by formulating a NP that is loaded with an active component and is also coated with a specific ligand and an imaging agent. The ligand can bind to the targeted area like a tumor or an organ. The imaging agent visualizes the arrangement of the NPs which can be used to monitor the treatment and the tissue development.

As the Nanoparticles are degraded, they release their load right at the requested site. An example could be a PLGA NP loaded with a chemotherapeutic and coated with a tumor specific antibody and Quantum Dots. Theranostics can also include environmentally responsive drug release like heat sensitive liposomes (Lammers et al. 2011).

PLGA NPs are well suited for drug delivery. Many therapeutic compounds such as peptides, macromolecules or nucleic acids show low bioavailability when administered orally due to degradation processes or a lack of suitable transporters in the digestive system. Even after i.v. injection there is the issue of degradation by enzymes which can be rapid for many peptides. Another problem is the clearance in liver, spleen and kidneys, or the opsonization by the immune system. The blood-brain barrier which is not permeable for the majority of drugs available today posts another boundary. To avoid these problems, drug carriers can be used for a variety of different applications. The sustained release of the embedded drug load allows longer intervals between injections. Coating with specific ligands enables targeting. This way the drug is used more efficiently because it can be trapped at the site of action, hence reducing side effects in heathy tissues (Makadia and Siegel 2011). *In vivo* tracking of theranostic NPs could be a powerful tool for non-invasive diagnostics.

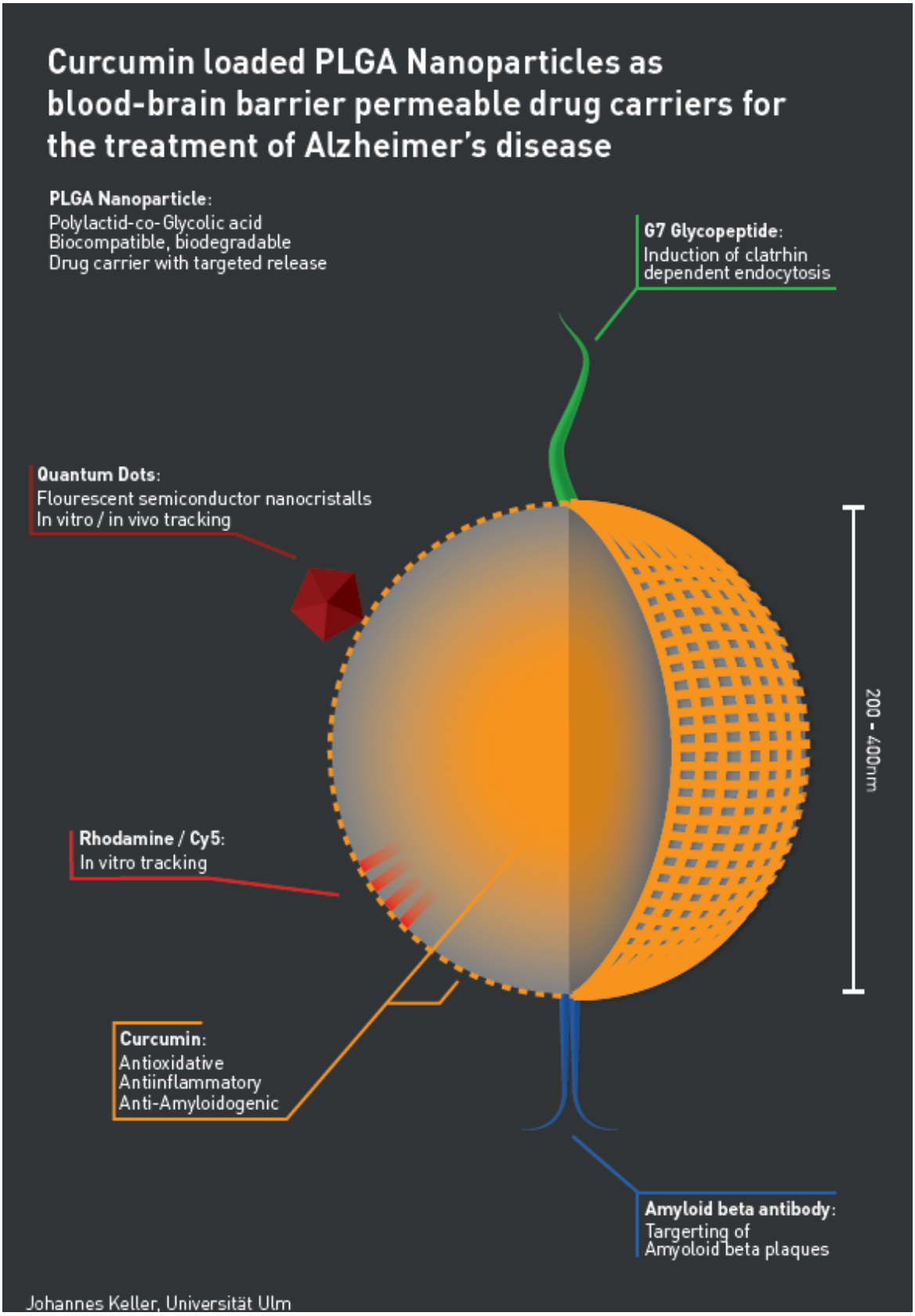


Figure 4: Schematic visualization of Nanoparticle modifications

1.10. Aims of the Thesis

The main risk factor for AD is the age of the patient. This implies that our aging population will face more and more cases of AD, as our average life expectancy increases. The limitations caused by AD are not just devastating for the patient and its family, but there is also a big economic factor, because the disease usually results in dependency on caregivers.

The treatment today is entirely symptomatic with acetylcholinesterase inhibitors like "Donepezil" or NMDA-antagonists like "Memantine". There is no causal therapy, no screening tool and no preventive measure. The characteristics of AD spell many difficulties for possible treatments. First, there is the blood-brain barrier stopping many chemicals from entering the central nervous system. Then, there is the aspect that it often takes decades of pathological Aβ accumulation before symptoms appear. This shows the need for better prevention and diagnostics that must be well tolerated. Curcumin is a promising compound with the limitation of poor oral bioavailability and limited uptake into the CNS. The g7-NP-Cur can improve the efficacy by masking the transported compound and by initiating the crossing of the BBB.

The first main aim of this thesis is to show that Curcumin loaded PLGA NPs with the mentioned modifications do not have toxic effects on primary hippocampal rat neurons *in vitro*. The safety of AD medications should be the main concern since the administration will usually be required over many years. The disease does also not pose an immediate threat to the patient's life, making aggressive treatment ethically unacceptable. AD treatment should be relatively risk-free and well-tolerated.

The second aim is to investigate the beneficial effects of the Curcumin after its release from the NPs. The anti-inflammatory, antioxidative and anti-amyloidogenic effects are to be proven. It will be investigated if NP-Cur can reduce the Aβ burden. It will be tested if this reduction of Aβ aggregates results in less of an inflammatory response. Next it will be shown if the possible anti-inflammation could cause a decrease in oxidative stress. A reduction of ROS could mean improved cell viability not just in AD but in many other cases of chronic inflammation that results in oxidative stress.

The third aim is to show the potential benefit of drug loaded NPs. It is to be shown if the tested NPs are taken up into neurons and if the Curcumin load will co-localize with the Aβ plaque that it is meant to target. The fate of the NPs after the injection is crucial to estimate their local concentration and effectiveness. The application of free Curcumin and Curcumin loaded NPs will be compared.

The NP formulations in this study aim to be used as theranostic medicine in the long term. Theranostic NPs include both diagnostic and therapeutic potential. AD is a chronic disease that persists for many years. Theranostic NPs can deliver their drug in a sustained fashion maintaining the dosage over a longer interval. The visualization of the NP localization can be used to evaluate the development of the targeted tissue.

2. Materials and Methods

2.1. Materials

2.1.1. Chemicals

Acrylamide (30%)	SERVA Electrophoresis GmbH, Germany
Amyloid Beta 1-42	American Peptides, USA/ AnaSpec, USA
APS - Ammonium persulfate	Sigma-Aldrich, USA
B27 supplement	Invitrogen, USA
Bromophenol blue	Sigma-Aldrich, USA
Chloroform	J.T. Baker®, USA
Coomassie brilliant blue (powder)	SERVA Electrophoresis GmbH, Germany
Complete Mini, EDTA Free	Roche, Germany
Curcumin	Sigma-Aldrich, USA
DAPI - 4,6-di-amino-2-phenylindol	Thermo Scientific™, USA
Dimethylarsinic acid sodium salt trihydrate	Millipore, USA
Dulbecco´s modified eagle´s medium	Gibco®, USA
D(+)-Saccharose	Carl Roth®, Germany

Dithiothreitol (DTT)	Sigma-Aldrich, USA
DL-Glutaraldehyde	Sigma-Aldrich, USA
Ethanol	Sigma-Aldrich, USA
FCS – fetal calf serum	GE Healthcare Life Sciences, USA
Glutamine	Gibco®, USA
Glycerol	Sigma-Aldrich, USA
Glycine	Sigma-Aldrich, USA
Hepes	Carl Roth®, Germany
Isopropanol	Pharmacy, Ulm University
HBSS - Hanks' Balanced Salt Solution	GE Healthcare Life Sciences, UK
Hydrochloric acid solution	Sigma-Aldrich, USA
Milk powder	Sigma-Aldrich, USA
Methanol	Sigma-Aldrich, USA
Neurobasal medium	Gibco®, USA
10X PBS	Life technologies, USA
Penicillin-Streptomycin	Invitrogen, USA
Pierce ECL western blotting substrate	Thermo Scientific™, USA
Phosphoric acid	Merck, KGaA, Germany
Poly-L-lysin (PLL)	Sigma-Aldrich, USA
TEMED	Bio-Rad, Germany
Tris buffer	Applichem, Germany

SDS ultrapure	Carl Roth®, Germany
Sodium Chloride	Sigma-Aldrich, USA
Triton® X-100	Sigma-Aldrich, USA
Trypsin	Invitrogen, USA
Tween-20	Carl Roth®, Germany
VectaMount™ AQ	Vector Laboratories, USA

2.1.2. Buffers

Buffer A	1% (v/v) 5 M Hepes (pH 7.4) 16% (v/v) 2 M Sucrose
Running buffer (10X)	14.4% (w/v) glycine 1% (w/v) SDS 3.03% (w/v) Tris in ddH_2O stored at room temperature

SDS sample buffer (4X)	25% (v/v) 1 M Tris-Cl pH 6.8 8% (w/v) SDS 60% (v/v) 50% Glycerol 4.6% (w/v) DTT 0.02% (w/v) Bromophenol blue in ddH_2O
Transfer buffer (10x)	14.4% (w/v) glycine 3.03 % (w/v) Tris in ddH_2O
Tris Buffered Saline (TBS, 10X)	2.42% (w/v) Tris 5.84% (w/v) NaCl in ddH_2O pH 7.5

2.1.3. Solutions

Ammonium persulfate	10% (w/v) APS in ddH_2O
Blocking solution	10% (v/v) FCS in PBS long-term storage at -20°C

Bradford reagent	10% (v/v) phosphoric acid 5% (v/v) Ethanol 10% (w/v) Serva Blue in ddH_2O
DAPI solution	0.2 µg/ml in PBS
Fixative (PFA-Sucrose)	4% (w/v) PFA 4% (w/v) sucrose in 1X PBS long-term storage at -20°C
Fixative (for perfusion)	2% PFA, 2.5% Glutaraldehyde 1% Sucrose 0.1M Cacodylate buffer in ddH_2O
Milk, Blocking Solution	5% (w/v) milk in 0.05% TBST Centrifuge at 10,000 rcf, 30 minutes, 4°C supernatant stored at -20°C

1X PBS	10% (v/v) 10X PBS in ddH_2O
Poly-L-lysin (PLL)	0.1 mg/ml PLL in HBSS sterile filtered before use
SDS	10% (w/v) SDS in ddH_2O
Sodium Chloride, 150mM	0.87% (w/v) NaCl in ddH_2O
Sucrose, 2 M	68.4% (w/v) Sucrose in ddH_2O
1 M Tris HCl	12.14% (w/v) Tris in ddH2O, pH 6.8
1.5 M Tris HCl	18.17% (w/v) Tris in ddH_2O, pH 6.8

Triton, permeabilization buffer	0.2% (v/v) Triton® X-100 in 1X PBS
TBST, (Washing buffer)	0.2% (v/v) Tween® in 1X TBS
TBST (Antibody incubation buffer for WB)	0.05% (v/v) Tween® in 1X TBS

2.1.4. Kits

Table 1: Kits used for the Aβ aggregation assay, cell viability assays and the detection of oxidative stress.

Kit	**Company**
Thioflavin T β - Amyloid (1 - 42) Aggregation Kit	SensoLyte
Apoptotic/Necrotic/Healthy Cell Detection Kit	Promokine
CellRox® Green Reagent	ThermoFisher

2.1.5. Antibodies

Table 2: Antibodies used for immunocytochemistry, Western blots and Dot Blot.

Antibody	**Company**	**Dilution**	**Species**
Anti-Actin B	Sigma-Aldrich	1/10000	Mouse
Anti-Amyloid Beta	Sigma-Aldrich	1/1000	Rabbit
Anti-Caspase 3	Cell Signaling Technologies	1/500	Rabbit
Anti-Clathrin	Abcam	1/1000	Rabbit
Anti-Homer	Abcam	1/500	Rabbit
Anti-IkappaB alpha	Biorbyt	1/500	Rabbit
Anti-Map 2	Synaptic Systems	1/800	Mouse
Anti-Mouse	Dako	1/10000	Goat
Anti-Mouse Alexa 432	Abcam	1/500	Goat
Anti-Mouse Alexa 488	Abcam	1/700	Goat
Anti-PARP	Cell Signaling Technologies	1/1000	Rabbit
Anti-PSD 95	Abcam	1/1000	Mouse
Anti-Rabbit	Dako	1/1000	Swine
Anti-Rabbit Alexa 647	Abcam	1/500	Goat
Anti-Shank 3	Abcam	1/500	Rabbit

2.1.6. Nanoparticle components

Curcumin:

IUPAC Name: (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione

Alternative name: Diferuloylmethane

Chemical sum formula: $C_{21}H_{20}O_6$

Company: Sigma-Aldrich

CAS number: 458-37-7

Quantum Dots:

Commercial name: Qdot 605 ITK Amino (PEG) Quantum Dots

Chemical formulation: CdSe, ZnS

Company: Molecular Probes, Q21501MP

PLGA – poly(lactide-co-glycolic-acid):

Commercial name: PLGA RG 503-H

Sum formula: $[C_3H_4O_2]_x\,[C_2H_2O_2]_y$

Molecular weight: 24.000-38.000 g/mol

PLA : PGA ratio: 50:50

Company: Sigma-Aldrich

CAS number: 26780-50-7

PVA – polyvinyl alcohol:

Sum formula: $(C_2H_4O)_n$

Molecular weight: 15.000 g/mol

Company: Fluka

CAS number: 9002-89-5

Anti-Amyloid Beta 13-28 antibody:

Company: Sigma- Aldrich, 2 mg/ml, mouse

Rhodamine:

Company: Sigma-Aldrich

CAS number: 509-34-2

Cyanine–5–amine:

Company: Lumiprobe USA

CAS number: 1469277-96-0

G7 glycopeptide:

AS-sequence: H2N-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-β-D-Glucose)-CONH2

Company: Mimotopes

2.2. Equipment

2.2.1. General laboratory equipment

Analytical balance	Sartorius, Germany
Autoclave (Systec DX-100)	Systec GmbH, Germany
Blotting systems (Wet/Tank; Mini Transblot®)	Bio-Rad, Germany
Centrifuge (Benchtop, ventilated)	Eppendorf, Germany
Centrifuge (ultra-centrifuge, Avanti™ J25)	Beckman Coulter, USA
Centrifuge Biofuge Freso (Benchtop, refrigerated)	Thermo Scientific™, USA
Chemiluminescence system (Microchemi 4.2)	Biostep GmbH, Germany
CO_2 Incubator (37°C)	Thermo Scientific™, USA
Emmi-05P Sonicator	Emag, Germany
Freezer (-20°C)	Liebherr, Switzerland
Freezer (-80°C)	Thermo Scientific™, USA
Fridge	Liebherr, Switzerland
Ice machine	Ziegra Eismaschinen, Germany
Laboratory glassware washer	Gemini Techniek BV, Netherlands
Microplate reader (Multiskan RC)	Labsystems, USA
Microplate reader (Tecan Infinite PRO 200)	Tecan, Switzerland
Microscope Axioskop 2 mot plus (fluorescence)	Zeiss, Germany
Microscope Axiovert 40 CFL (inverted)	Zeiss, Germany

Microscope Stemi 200 CS (stereo)	Zeiss, Germany
Microtome (Cryostat, CM 3050S)	Leica, Germany
Microtome (Vibrating blade)	Thermo Scientific™, USA
RotorGene Q System	Qiagen, Germany
Sonicator (Bath Sonicator)	Branson Ultrasonics Corp, USA
Sonicator (Pulse Sonicator, Sonopuls)	Bandelin, Germany
Spectrophotometer (Nanodrop™ 2000)	Thermo Scientific™, USA
Steam sterilizer	Biomedis, Germany
Sterile bench (MSC-Advantage)	Thermo Scientific™, USA
Tank blot machinery	Bio-Rad, Germany
Test tube rotator	Snijders, Netherlands
Thermomixer	Eppendorf, Germany
Ultra-Centrifugal Filter	Amicon, Germany
Vortex Mixer	Scientific Industries, USA
Water bath	Thermo Scientific™, USA

2.2.2. Labware

Beakers	Duran®, Germany
Cell culture dish (10cm Ø)	Becton Dickinson and Company, USA
Cell culture plate (flat bottom, 24 well)	Becton Dickinson and Company, USA
Cell culture plate (flat bottom, 96 well)	Sigma-Aldrich, USA
Cell scraper	Sarstedt, Germany
Cell strainer (125 µm, 70 µm)	Becton Dickinson and Company, USA
Cover glass (rectangular, Ø 13 mm)	VWR®, USA
Coverslips (round)	VWR®, USA
Cylinders (Single scale, Graduated)	VWR®, USA
Falcon tubes	Sarstedt, Germany
Filter (bottle top, 0.45 µm)	Sarstedt, Germany
Filter (syringe filter, 0.2 µm)	Sarstedt, Germany
Flasks	Duran®, Germany
Gloves (sempercare)	Sempermed, Austria
Microscopic slides	VWR®, USA
MilliQ water system	Millipore, USA
Neubauer counting chamber	Hecht Assistant, Germany
Nitrocellulose membrane	Amersham, UK
Pipetboy	Integra Bioscience, Germany
Pipette (10 µl, 200 µl, 1000 µl, 5000 µl)	AHN Biotechnology GmbH, Germany

Pipette (300 µl Multichannel)	Eppendorf, Germany
Pipette (5, 10, 25 ml)	Becton Dickinson and Company, USA
Pipette tips (10 µl, 200 µl, 1000 µl, 5000 µl)	Sarstedt, Germany
Reaction tubes (0.5, 1.5, 2 ml)	Sarstedt, Germany
Reagent bottles	VWR®, USA
SDS-PAGE glass plates (7.3x10.1cm)	Bio-Rad, Germany
SDS-PAGE mini-protean comb	Bio-Rad, Germany
Surgical disposable scalpels	B. Braun, Germany
Surgical instruments	Dumostar®-Biology, Switzerland
Syringe (1 ml)	B. Braun, Germany
Syringe (10, 50 ml)	Becton Dickinson and Company, USA
Whatman filter papers 3 mm	Schleicher and Schuell, Germany

2.2.3. Equipment for Nanoparticle formulation and characterization

Atomic Force Microscope: Park Instruments, Sunnyvale, USA

Dynamic Light Scattering detector: Malvern Zetasizer, Nano – ZS, UK

Electron Spectroscopy for Chemical Analysis: XRC 1000 X-ray source analysis system with a Phoibos 150 hemispherical electron analyzer. (Specs Surface Nano Analysis, Germany)

High Performance Liquid Chromatography detector: JASCO Model 7725i, Cremella, Italy

Lyophilizer: Heto Lyo Lab 3000, SelectScience, UK

Sonication bath: Sonorex RK 100H, Germany

Sonication probe: Branson SLPe OptoLab, Italy

Transmission Electron Microscope: JEM 2010; JEOL, Peabody, USA

2.3. Methods

2.3.1. Preparation of primary hippocampal cell cultures

Pregnant rats were purchased from Janvier Labs. All animal experiments were performed in compliance with the guidelines for the welfare of experimental animals issued by the Federal Government of Germany and approved by the Regierungspraesidium Tuebingen and the local ethics committee at Ulm University (Ulm University, ID: O.103). Primary hippocampal cell cultures were prepared from fetal rat brains at embryonic day E18. The brains were collected in pre-chilled Hanks' Balanced Salt Solution (HBSS) and the hippocampi were dissected. The cells in 1800 µl HBSS were then trypsinized with 200 µl Trypsin 2.5% for 20 min at 37°. After 5 washing steps, cells in 1600 µl HBSS were incubated with 400 µl DNAse 0,01% for a few seconds. The suspension was filtered through a 125 µm sieve and incubated with 18 ml of DMEM (Dulbecco's Modified Eagle Medium), 10% FCS, 1% glutamine and 1% penicillin-streptomycin. Cells were then counted in the Neubauer-Chamber. 24-well plates and 10 cm petri dishes were treated with poly-L-lysine (PLL). $3x10^4$ cells were plated on every cover glass in the 24-well plates, and $3x10^6$ cells on every 10 cm petri dish. After overnight incubation at 37°C and 5% CO_2 the culture medium was exchanged with Neurobasal medium. This was supplemented with B27 (Invitrogen), 0.5 mM L-Glutamine (Invitrogen) and 100 U/ml penicillin-streptomycin (Invitrogen) and maintained at 37°C in 5% CO_2.

2.3.2. Treatment of hippocampal cells

Nanoparticles were diluted in 1 ml of HBSS (Hanks' Balanced Salt Solution) and then sonicated using the Emmi-05P Sonicator. Diluted Nanoparticles were stored at -20°C and used within a few weeks.

Curcumin from Sigma Aldrich was diluted to 1 mM in DMSO and then stored at -20°C.

Synthetic Aβ peptides - Aβ (1-42) were purchased from American Peptides and Anaspec. The peptides were initially dispersed in ddH_2O at a concentration of 1 mg/ml and then stored at -20°C until used. Further dilutions of the peptides were performed in the culture medium.

2.3.3. Immunocytochemistry

For detection of fluorescence via immunocytochemistry, the primary cultures were fixed with 4% paraformaldehyde (PFA)/ 4% sucrose/ PBS at 4°C for 20 min. After washing 3 x 5 min with 1X PBS containing 0.2% Triton X-100 at RT, blocking was performed with 10% FCS/ 1X PBS for 1 h at RT. Subsequently, samples were incubated with the primary antibody at RT for 2 h or at 4°C overnight under agitation. After 3 x 5 min washing steps with 1X PBS, incubation with the secondary antibody coupled to Alexa488, Alexa568 or Alexa647 followed for 1 h. There were 3 final washing steps with 1X PBS for 5 min. Cell nuclei were counterstained with DAPI and coverslips were mounted using Vecta Mount (Vector Laboratories).

2.3.4. Detection of healthy, apoptotic and necrotic cells

Toxicity of the treatment was evaluated by a healthy/apoptotic/necrotic cell detection kit using primary hippocampal rat cultures after 14 days in vitro (DIV 14) on 24-well plates. For a positive control apoptosis/necrosis was induced with 500 µl of ethanol 70%. Cells were washed with 1X binding buffer. Cells were incubated with a staining solution containing 100 µl of 1X binding buffer, 5 µl of FITC-Annexin V, 5 µl of Ethidium Homodimer III and 5 µl of Hoechst 33342 for 15 min at RT protected from light. Two more washing steps with 1X binding buffer followed. Then cells were fixed with PFA and CaCl 1.25 mM for 15 min. The coverslips were mounted on microscopic slides with a drop of Vecta Mount.

2.3.5. Preparation of cell lysates

Treated primary hippocampal rat cultures at DIV 14 were scraped of petri dishes and homogenized in lysis buffer (150 mM NaCl, 1% Triton® X-100, 50 mM Tris HCL, pH 8.0) containing protease inhibitor (Roche, Mannheim, Germany) for 2 h under agitation. From this homogenate cell debris and nuclei were removed by centrifugation at 3200 rpm for 10 min at 4°C resulting in supernatant S1 fraction and pellet P1 fraction.

2.3.6. Ultra-centrifugation of Amyloid Beta

To detect the fibrillation state and concentration of the Aβ in the supernatant ultra-centrifugal filters from Amicon were used to increase the sample concentration. 500 µl of the supernatant were loaded into a filter and then spun with 14000 rpm for 10 minutes. The filter was then placed upside down in a new tube and spun again with 1000 rpm for 10 minutes.

2.3.7. Thioflavin T Beta-Amyloid (1-42) Aggregation Kit

The Thioflavin T Beta-Amyloid (1-42) Aggregation Kit was purchased from SensoLyte. 900 µl of the assay buffer were added to 100 µl of the Thioflavin to get a 2 mM solution. The included Aβ peptide was diluted in 1 ml of cold assay buffer and sonicated for 5 minutes. Then it was transferred into a tube and centrifuged at 10,000 rpm for 5 minutes at 4°C.

To set up the fibrillation reaction 10 µl of Thioflavin 2 mM were added into each well. Then 85 µl of the Aβ solution and 5 µl of the test compounds or control were added. As a control for Curcumin we used DMSO since it was used to dilute the Curcumin, as well. The aggregation inhibitor supplied in the kit was used as negative control. A blank control was established using only assay buffer.

The fluorescence intensity at an emission of 484 nm was measured at 37°C with an excitation of 440 nm. The measurements were repeated every 10 minutes for up to three hours using a microplate reader (Tecan Infinite PRO 200, Tecan Switzerland).

For analysis the blank control was set as background fluorescence and subtracted from the other readings. The readings are expressed as relative fluorescence units and plotted versus fibrillation time.

2.3.8. Fenton's reaction to induce oxidative stress

Oxidative stress was introduced *in vitro* to establish AD like conditions and to obtain a positive control for the CellROX® oxidative stress detection kit (Thermo Fisher). The kit was used to show possible antioxidant effects of Curcumin and the NP-Cur *in vitro* on primary hippocampal rat neurons at DIV 14.

Fenton's reagents were used to introduce oxidative stress to the cell culture. 0.5 µM of $FeCl_2$ and 2.5 µM of H_2O_2 inflicted significant oxidative stress upon cells on a 24 well plate overnight. The dissociated Fe^{2+} ions are oxidized while reducing H_2O_2 to one Hydroxide Ion and one Hydroxyl radical:

$$\mathbf{Fe^{2+} + 2Cl^- + H_2O_2 \rightarrow Fe^{3+} + 2Cl^- + \cdot OH + OH^-}$$

The cells were preincubated with the Fenton's reagents for one day. Curcumin and Curcumin loaded NPs were added to investigate the antioxidative effects.

2.3.9. CellROX® oxidative stress detection

CellROX® oxidative stress green reagents (Thermo Fisher) were used to evaluate the antioxidant properties of Curcumin and NP-Cur on primary hippocampal rat neurons at DIV 14. Cells on a 24 well plate were treated with the test compounds or left as control. Then the CellROX® green reagents were added at a concentration of 5 µM after a NP incubation of 30 minutes, 1 day or 7 days at 37°C. The medium was removed, and the cells were washed 3 times with PBS. Next, the cells were fixed in formaldehyde for 15 minutes. The fluorescence was measured using a fluorescence reader to characterize the antioxidant effects. The CellROX® green reagent emits light at 520 nm being in its oxidized form while it shows significantly less fluorescence in the reduced state.

2.4. Western blotting

Bradford Assay:

To determine the protein concentration of the samples Bradford assays have been performed. The dye "Coomassie Brilliant Blue G-250" builds complexes with protein side chains. By doing so it is stabilized in its anionic blue conformation. This was quantified in a 96 well plate with a multiplate reader. 40 µl of Bradford solution and 4 µl of 150 mM NaCl were added to each well. 1 µl of the cell lysates were added to the according wells. A blank was obtained by adding 1 µl of the lysis buffer instead. All the values were normalized with a standard curve of BSA (Bovine Serum Albumin).

Gel casting:

Proteins were separated using a 15% SDS-Page.

The resolving polyacrylamide-gel was formulated by mixing the following solutions:

50%: Acrylamide/Bis-acrylamide solution of 30% concentration

25%: 1.5M Tris (tris(hydroxymethyl)aminomethane) solution (pH 8.8)

1%: SDS (sodium dodecyl sulfate) of 10% concentration

1%: APS (ammonium persulfate) of 10% concentration

0.05%: TEMED (Tetraacetylethylenediamine)

The stacking gel was obtained by mixing:

17%: Acrylamide/Bis-acrylamide solution of 30% concentration

25%: 1.5M Tris solution (pH 8.8)

12.5%: 1M Tris (pH 6.8)

1%: SDS (sodium dodecyl sulfate) of 10% concentration

1%: APS (ammonium persulfate) of 10% concentration

0.05%: TEMED (Tetraacetylethylenediamine)

The resolving gel was poured in the glass cassette sandwich and the surface was overlaid with isopropanol to ensure an even surface and to exclude oxygen. After polymerization, the isopropanol was poured out, rinsed with ddH_2O and then dried of using filter paper. Then the stacking gel was poured on top of the resolving gel and a 10-20 well comb was placed in it. The gel was stored at 4°C and used within a week.

Gel electrophoresis:

Bradford assays were performed to determine the amount of sample containing 10 µg of the investigated protein. The sample was mixed with a 4X SDS sample buffer, then spun down, then heated at 90°C for 15 minutes, then spun down again. The mixture was loaded into the polyacrylamide gel and the electrophoresis chamber was filled with 1X running buffer. 100 V were used for the beginning of the electrophoresis until the front reached the resolving gel. Then the Voltage was increased to 210 V until the running front nearly reached the bottom of the cassette.

Gel transfer:

A Wet-Blot-Sandwich was used to transfer the protein onto a nitrocellulose membrane. Gel and membrane were placed in between 4 filter papers and surrounded by sponges. This sandwich was put into the blotting cassette after ensuring no bubbles had formed. The blotting chamber was filled with transfer buffer and the sandwich was placed in it. The transfer was performed using 90 V for 90 minutes at 4°C.

Protein detection:

The nitrocellulose membrane was blocked using 5% milk for 1 h at RT. Then it was incubated with the primary antibody on a shaker for 2 h at RT or overnight at 4°C. Three washing steps using 0.05% TBST for 15 minutes followed. Then the membrane was incubated with the secondary antibody for 1 h. Three more washing steps with 0.2% TBST were performed. The immunoreactivity was visualized with HRP (Horseradish Peroxidase) conjugated secondary antibodies and the Super Signal detection system (Pierce, Upland, USA) using a chemiluminescence western detection system (Biostep).

Western blot quantification:

For Western blot quantification the evaluation of the band signal was obtained using ImageJ. Three independent experiments were performed. The individual bands were selected, and the integrated density was measured. Bands were normalized to β-actin and the ratios were averaged.

2.5. Nanoparticle preparation

The Curcumin loaded Nanoparticles were formulated by the Solvent Evaporation Method in a single emulsion process.

50 mg of the PLGA (PGA:PLA ratio 50:50) were dissolved in 1.5 ml of DCM - dichloromethane. 2.5 mg of Curcumin were added to the solution. The polymer drug solution was then emulsified in 6 ml of purified water with 2% PVA – polyvinyl alcohol as emulsifier. This was performed by a sonication probe with a power of 55 watts for 60 sec. The DCM was evaporated under the hood over 3 h with permanent steering. After centrifugation with 16000 rpm for 10 min at 4°C the supernatant was discarded. The pellet which now contains the Curcumin loaded Nanoparticles was dissolved in 4 ml of purified water and then sonicated in the water bath sonicator for 5 min. Size, polydispersity index and Zeta potential of the produced NPs were measured via dynamic light scattering (Malvern Zetasizer Nano – ZS) before and after the centrifugation.

2.5.1. Purification and collection of the NPs

All NP preparations were purified by an ultracentrifugation process carried out at 17,000 rpm for 10 min (4°C; Sorvall RC28S, Dupont, Brussels, Belgium) to remove the un-formed material and the free surfactant fraction. The NPs were washed three times with water, resuspended in water (5 mL) and freeze-dried (-60°C, 1x10-3 mm/Hg; LyoLab 3000, Heto-Holten) by using D-(+)-trehalose (Fluka–Sigma–Aldrich) as cryo-protectant (1:1 w/w polymer/trehalose ratio). The yield (Yield%) was calculate as follows:

Yield (%) = Sfd/Ctot x100

Sfd is the amount (mg) of freeze dried samples (excluded residual PVA and anhydrous trehalose) and Ctot is the total amount (mg) of polymer and Cur weighted for the preparation.

2.5.2. Surface modifications of NPs

22.5 mg of NPs were diluted in 1.5 ml of MES - 2-(*N*-morpholino)ethanesulfonic acid buffer (Sigma Aldrich) with a concentration of 0.1M and a pH of 4.7. The solution was added to 23 mg of NHS - N-hydroxysuccinimide (Sigma-Aldrich) and 69 mg of EDC - 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich), to activate the carboxylic groups of the polymer. After 1 h the NPs were recollected by centrifugation with 16000 rpm for 10 min at 4°C. Then they were resuspended in 2 ml of PBS with a pH of 7.4. Next, 1.5 ml of Nanoparticle solution containing about 15 mg of NPs were incubated with 40 pmol of PEGylated Quantum Dots. The incubation was performed at RT for 1 h under agitation.

For the surface modification with the anti-Aβ antibody the same protocol was applied as for the Quantum Dots. 1.5 ml of Nanoparticle solution containing about 15 mg of NPs were incubated with 40 pmol of anti-Aβ antibody. (Belletti et al. 2015).

Polymer modification with Rhodamine, Cyanine-5-amine and g7

The two different red dyes Rhodamine and Cy5 – Cyanine-5-amine and the opioid analogue g7 were already added into the polymer mix before the NPs were formulated. They were added to the pure PLGA polymer to reach a concentration of 2% for Rhodamine, 5% for Cy5 and 10% for g7. The rest of the formulation process was continued as described.

2.5.3. NP characterization

The Zetasizer (Nano ZS 3600, Malvern, UK; Laser 4 mW He–Ne, 633 nm, Laser attenuator Automatic, transmission 100–0.0003%, Detector Avalanche photodiode, Q.E. > 50% at 633 nm) was used for characterization of mean diameter, PDI – polydispersity index, and Zeta potential of the NPs. The Zeta potential was measured using the same equipment with a combination of a laser Doppler velocimetry and phase analysis light scattering (PALS). All the data are the means of three determinations carried out for each preparation lot.

2.5.4. Determination of drug loading capacity and encapsulation efficiency

Cur loaded into g7-NPs-Cur was extracted and quantified by high performance liquid chromatography (HPLC). 1 mg of NPs was solubilized into 0.5 mL of chloroform by using a bath sonicator for 5 min. Then, 4 mL of isopropylic alcohol were added to precipitate the polymer. The mixture was filtrated through PTFE filter (0.2 mm, Sartorius, Firenze, Italia) and the solution analyzed by HPLC. The HPLC apparatus (JASCO Europe, Cremella, Italy) comprised a Model PU2089 pump provided with an injection valve with a 50 µL sample loop (Model 7725i, Jasco) and the UV/VIS detector (UV1575, Jasco). The analysis was performed in isocratic conditions, at a flow rate of 1 mL/min and at r.t. on a column HC-C18 (250mm x 4.60 mm, 5 µm, Agilent Technologies) equipped with a security guard. The mobile phase was a mixture 41:23:36:1 (v/v/v/v) of acetonitrile, methanol, water and acetic acid. Chromatographic peak areas of the samples were recorded and analyzed using a JASCO software (JascoBorwin 1.5) and the concentration was calculated using the calibration curve previously set up (linearity was assumed in the range 0.3–9.5 µg/mL; r2 = 0.9951). The encapsulation ability of NPs was evaluated considering the encapsulation efficiency (EE%) and the loading capacity (LC%) calculated using the following equations:

$EE\% = D/ Td \times 100$

$LC\% = D/ W \times 100$

where D is the amount of the encapsulated drug, Td is the amount of drug added for the preparation and W is the weight of NPs (polymer + drug).

All the data are expressed as the mean of at least three determinations.

2.5.5. Atomic Force Microscopy

Atomic Force Microscopy (AFM) was used to visualize the morphology of the samples. The observations were performed with an Atomic Force Microscope (Park Instruments, Sunnyvale, CA, USA) at about 20°C operating in air and in Non-Contact (NC) mode using a commercial silicon tip-cantilever (high resolution noncontact "GOLDEN" Silicon Cantilevers NSG-11, NTMDT, tip diameter=5–10 nm; Zelenograd, Moscow, Russia) with stiffness about 40 Nm^{-1} and a resonance frequency around 170 kHz. After the purification, the samples were dispersed in distilled water and applied on a freshly cleaved mica disk (1 cm×1 cm); 2 min after the deposition, the water excess was removed using blotting paper. The AFM images were obtained with a scan rate of 1 Hz and processed using a program obtained from Gwyddion (Department of Nanometrology, Czech Metrology Institute, Brno, Czech Republic). Images were flattened using second-order fitting to remove background curvature and slope from the images.

2.5.6. Transmission Electron Microscopy

Transmission Electron Microscopy (TEM) was used to complement the AFM images. TEM images enable the visualization of QDs as optical dense spots. The images were obtained by a TEM (model JEM 2010; JEOL, Peabody, MA, USA) operating at an acceleration voltage of 200 kV.

2.5.7. Electron Spectroscopy for Chemical Analysis

To identify the elements on the NP's surface ESCA was performed on a XRC 1000 X-ray source analysis system (Specs Surface Nano Analysis, Berlin, Germany) and a Phoibos 150 hemispherical electron analyzer (Specs Surface Nano Analysis), using $MgK\alpha_{1,2}$ radiations. The spectra were recorded in a fixed retardation ratio (FAT) mode with 40 eV pass energy. The pressure in the sample analysis chamber was ca. 10^{-9} mbar. The data were collected and processed using the SpecsLab2 software.

2.5.8. In vitro release studies

For each time point, 2 mg of g7-NPs-Cur were suspended in 1 mL of a 50% v/v of serum solution (FBS) at 37°C under electromagnetic stirring. After the incubation time (30 min, 3, 6, 24, 30, 48, 72 h), the suspensions were centrifuged (13,000 rpm for 15 minutes) to separate NPs (with possible adsorbed serum protein and residual loaded Cur) that precipitates from supernatant. The possible serum proteins adsorbed on NPs surface were quantified by colorimetric assay bicinchoninic acid (BCA) as described by manufacturer (Micro BCA protein assay kit composed of reagent A (alkaline tartrate-carbonate buffer), reagent B (bicinchonic acid solution) and reagent C (copper sulfate solution), purchased from Thermo Fisher Scientific Inc (Milan, Italy)) and subtracted from dried pellet to obtain the amount of NPs recovered after centrifugation.

2.6. Statistics

Statistical analyses were performed using Microsoft Excel and SPSS version 20. The data is shown as mean ± SEM. To compare groups, a one-way analysis of variance (ANOVA) was performed and then followed by the Bonferroni test, which is a post hoc test for comparison within groups.

Differences that where statistically significant are indicated in figures as * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

Immunocytochemistry: The acquisition and evaluation of all images were performed under blinded conditions. For each condition 10 optical fields were imaged. All signals within the measured optical fields were quantified in the following way: Fluorescence images were obtained with an upright Axioscope microscope equipped with a Zeiss CCD camera (16 bits; 1280x1024 ppi) using Axiovision software (Zeiss). Quantification was performed using ImageJ 1.49o.

3. Results

3.1. Physicochemical properties

3.1.1. NP size and surface potential

To measure size and surface potential each batch of Nanoparticles was characterized by dynamic light scattering. This method provides information regarding the mean diameter, the Zeta potential and the polydispersity index (PDI). As shown in Table 3 all average diameters are between 200-250 nm with a PDI lower than 0.15. This indicates a narrow size distribution range, favorable for systemic administration. Therefore, the chance of producing particles bigger than 500 nm is very unlikely. This is important since it has been described that particles bigger than 1000 nm are significantly more toxic than the smaller ones. The Zeta potential informs about the NP's surface potential. NPs that are empty or not coated with the g7 peptide are more negatively charged (-19 to -28mV) due to more accessible carboxylic groups on the NP's surface. G7 coated NPs show less negative Zeta potentials of around -13mV. This is still sufficient to cause repulsive forces between individual NPs to prevent massive aggregation in a hydrophilic medium. Rhodamine and Cy5 are dyes used for NP detection *in vitro*. The results indicate that the size and physicochemical properties of the NPs are in ranges that are expected to be well tolerated.

Table 3: Diameter, PDI and Zeta potential of the Nanoparticles. Dynamic light scattering was used to measure the mean diameter of all samples. The size distribution is shown as the polydispersity index (PDI). The NP's surface charge is called Zeta potential. Rhodamine and Cy5 are the dyes used for Nanoparticle detection *in vitro*. Performed by Tosi et al. in Modena with my contribution.

Nanoparticle	**Mean Diameter**	**PDI**	**Zeta potential**	**Dye**
NP	220 nm	0.13	-20 mV	Rhodamine
QD-NP	239 nm	0.11	-19 mV	Rhodamine
NP-Cur	235 nm	0.11	-19 mV	Rhodamine
QD-NP-Cur	250 nm	0.15	-28 mV	Rhodamine
G7-NP	202 nm	0.0325	-13 mV	Cy5
G7-Ab-NP	206 nm	0.089	-12.5 mV	Cy5
G7-NP-Cur	204 nm	0.075	-13 mV	Cy5
G7-Ab-NP-Cur	205 nm	0.089	-12,5 mV	Cy5

3.1.2. NP composition

To evaluate the concentration of the loaded Curcumin and the residual surfactant a HPLC (high performance liquid chromatography) was performed. The content of PVA (polyvinyl alcohol) was measured to be between 5-7% which is compatible with parenteral administration. The percentage of the loading capacity (LC) is the ratio of encapsulated drug out of the total mass of the NP. The loading capacity of 3.1% for the g7-NP-Cur is to be considered relatively high.

The encapsulation efficiency (EE) describes the ratio of encapsulated and total Curcumin. The percentage of yield expresses the ratio of the recovered freeze-dried sample out of the total mass of the sample. The ESCA (Electron Spectroscopy for Chemical Analysis) was performed to identify the elements on the NP's surface. The PLGA matrix is made up by the elements C (carbon), O (oxygen) and H (hydrogen). The N (nitrogen) that was detected on the surface of the g7 peptide coated NPs is proof of the successful surface engineering procedure. The results in Table 4 show that the NPs are composed mainly of the FDA approved PLGA as well as the g7 ligand and the Curcumin load. We conclude that the NP's size, charge, and composition are all suitable for systemic administration.

Table 4: Nanoparticle composition. HPLC (high performance liquid chromatography) was performed to determine the content of polyvinyl alcohol (PVA) as well as Curcumin. The loading capacity (LC) shows the percentage of Curcumin compared to the total Nanoparticle mass. The encapsulation efficiency (EE) specifies the amount of encapsulated Curcumin out of the total mass of Curcumin used. The percentage of Yield was expressed as the ratio of the recovered freeze-dried sample (excluded residual PVA) out of the total mass weighed (polymer and drug) in percent. ESCA: Electron Spectroscopy for Chemical Analysis identifies the composing elements. All the values represent the means of at least three experiments; standard deviation (SD) in parentheses. Performed by Tosi et al. in Modena with my contribution.

	NP	**G7-NP**	**G7-NP-Cur**
PVA (%) (+/− S.D.)	7 (2)	6 (2)	5 (3)
LC (%) (+/− S.D.)			3.1 (0.8)
EE (%) (+/− S.D.)			60 (6)
Yield (%) (+/− S.D.)	87 (2)	88 (3)	97 (2)
ESCA; C%, O%, N% (+/− S.D.)	77 (2) 22 (4) /	65 (3) 34 (2) 0.7 (0.1)	67 (3) 32 (3) 1.5 (0.1)

3.1.3. Fluorescence spectroscopy, Atomic Force and Transmission Electron Microscopy

To visualize the shape and surface morphology of the NPs Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) was used. Our average NPs of about 250 nm are smaller than the wavelengths of visible light which are between 400-750 nm. For this reason, it is physically impossible to present the NPs using a light microscope. In Fig. 5; 1. and 2. from a-d are AFM images. The NPs are in the same size range and they present a smooth surface. The aggregation of the NPs can partly be due to the freeze-drying process. Image 3. of b) and d) are TEM images. The optical dense spots are the Quantum dots on the NP's surface. The number c.3. displays the fluorescence spectroscopy of NP-Cur showing the emission spectrum of Curcumin loaded NPs without QDs. Curcumin has a broad emission range that changes depending on the solvent medium. Image d.4. shows how the QD-NP-Cur exhibit two emission peaks, from Curcumin at 510 nm and from the QDs at 608 nm. The excitation of Curcumin in these particular NPs with light of 405 nm results in an emission peak at 510 nm. The emission spectrum of other Curcumin formulations may vary. The QD signal is more specific with a narrow distribution range favorable for detection. The images show that most NPs are intact and in the same size range. Both modified and unmodified NPs show spherical morphology and a smooth surface with only little aggregation that is most likely caused by the dehydration process necessary for the analysis.

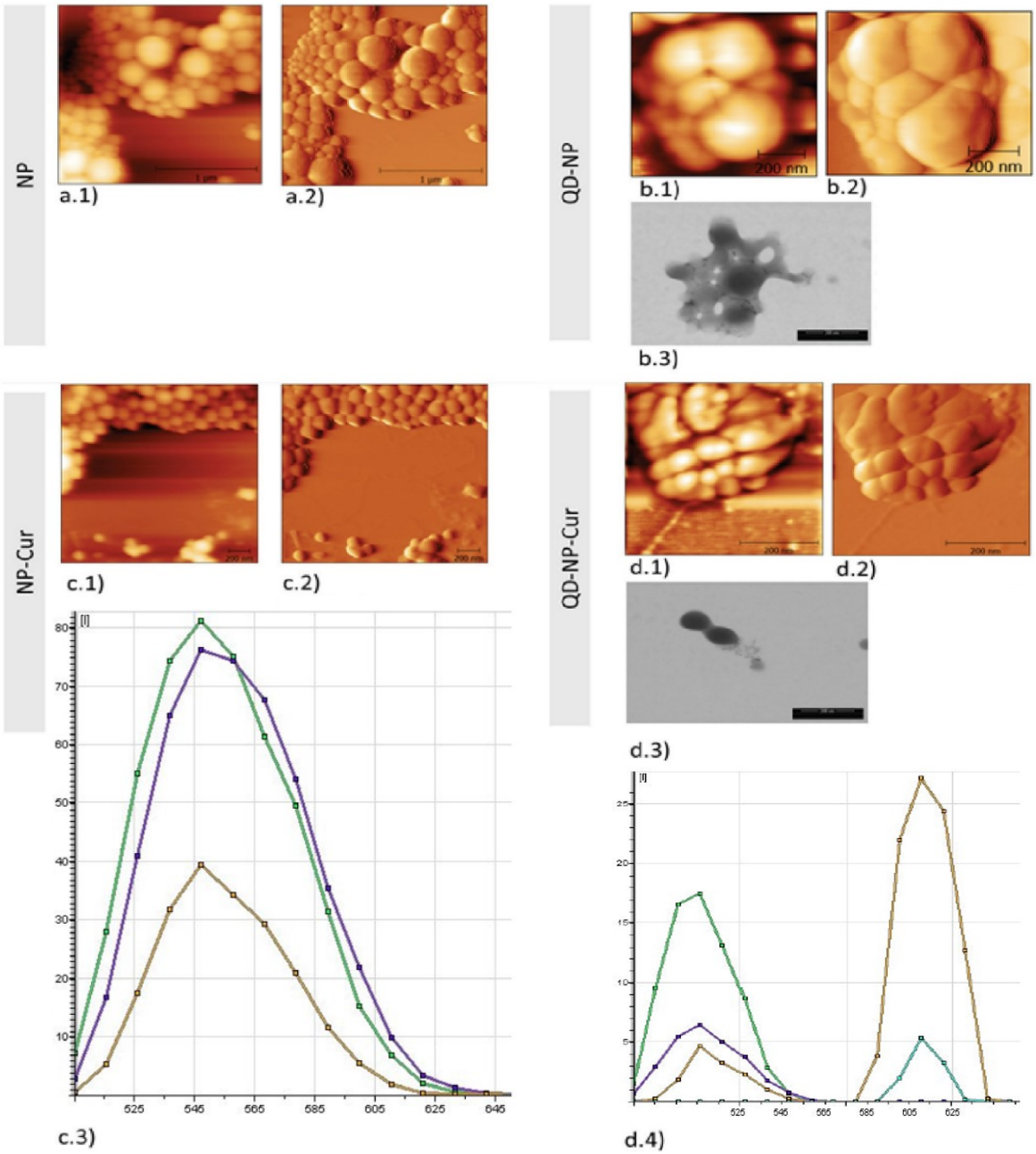


Figure 5: Atomic Force Microscopy, Transmission Electron Microscopy and Fluorescence spectroscopy of the investigated Nanoparticles (NPs). Images of loaded and unloaded NPs with and without Quantum Dots (QDs). a.1) + a.2) Atomic Force microscopic (AFM) images of the NPs. b.1) + b.2) AFM images of QD-NP. b.3) Transmission electron microscopic (TEM) images of the QD-NP. c.1) + c.2) AFM images of NP-Cur. The Fluorescence spectroscopy of the NP-Cur in c.3) shows the emission peak of NP-Cur at 545 nm. d.1) + d.2) AFM images and d.3) TEM image of QD-NP-Cur. d.4) Emission peaks of both the Curcumin at 510 nm and the QDs at 608 nm. Derived from (Barbara et al., 2017).

3.2. Curcumin Release Profile

In Fig. 6 it is demonstrated how much Curcumin will be released from the NP-Cur in which amount of time. High performance liquid chromatography was used to measure the release of Curcumin from the NP-Cur. Physiological conditions were simulated by using PBS as solvent medium. The initial burst of about 70% of the loaded Curcumin within the first 30 minutes can be explained with the formulation process. Firstly, not all Curcumin is inside the PLGA matrix, but also on the NP's surface. In addition, not all NPs are still intact at the end of the formulation process. After the initial burst, there was a sustained release over the next three days. The PLGA polymer used was 50% lactic and 50% glycolic acid. The PLGA ratio can be altered to create NPs with a longer half-life. The late release of about one third of the load shows that the NP-Cur are suitable for drug delivery with sustained release. The release profile is similar to a pharmaceutical application were a big loading dose is followed by smaller maintenance doses.

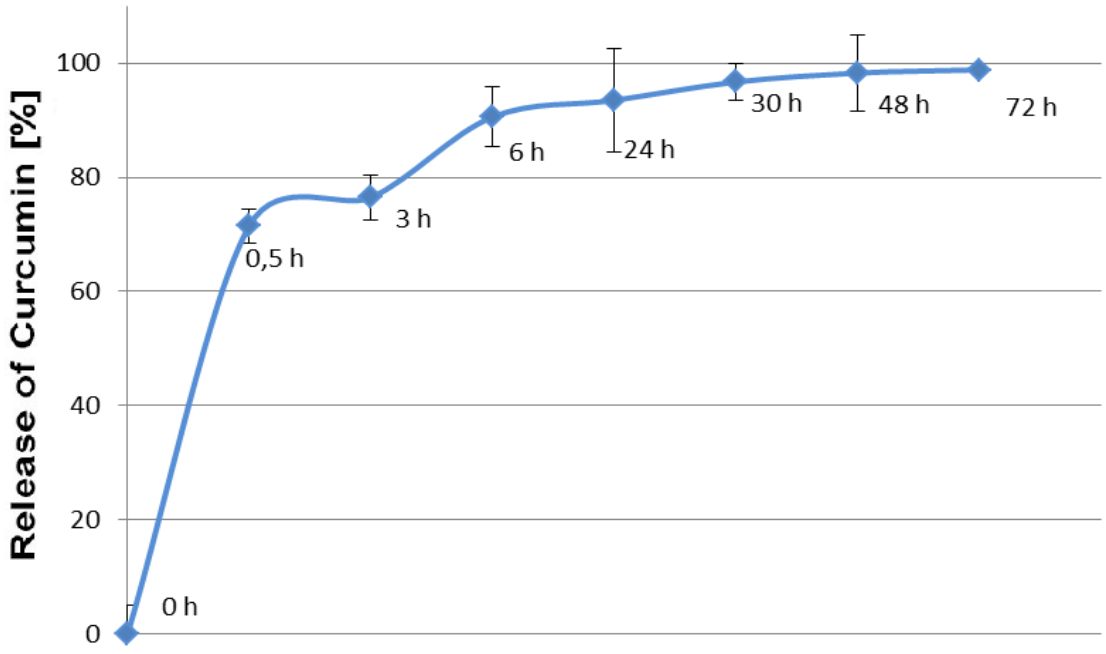


Figure 6: The Curcumin release profile of NP-Cur was obtained by high performance liquid chromatography of Nanoparticles in Phosphate-buffered Saline. The initial burst is followed by sustained release. Derived from (Barbara et al., 2017).

3.3. Cellular toxicity assays

To investigate whether the NPs induce apoptosis or necrosis, cell viability assays were performed using different concentrations and incubation periods.

Apoptosis is the process of programmed cell death and the bodies physiological way to recycle cells that are stressed, damaged or infected. The intrinsic pathway is activated when cells experience hypoxia or nutrient deprivation for instance. The extrinsic pathway is activated via the death receptor for example due to a viral infection. A certain rate of apoptosis is a normal process that does not involve inflammation. A strong increase in apoptotic nuclei can be a sign for pathological conditions.

Necrosis is the traumatic and less organized pathway of cellular death. It is induced by toxins, trauma or other kinds of severe cell damage. Necrosis is accompanied by inflammation and does not occur physiologically. Necrosis is usually a sign for compromising cellular damage that cannot be repaired.

All experiments concerning cell viability were performed on primary hippocampal rat neurons.

3.3.1. The NPs do not induce apoptosis *in vitro*

Cells on 24 well plates were incubated with 46-275 µg of different NPs for 24 hours. For the NP-Cur the total NP weight of 46, 115 and 230 µg resulted in Curcumin concentrations around 10, 20 and 40 µM. The Curcumin concentrations were intentionally chosen to be higher than the recommended 10 µM used in other studies. This way the results also represent higher concentrations that might occur if the NPs were to degrade faster. The experiments were performed on DIV 13 primary hippocampal rat neurons in cell culture. The DAPI staining was used to visualize the nuclei. Then apoptotic bodies were counted using a fluorescence microscope and ImageJ. Curcumin loaded NPs show a trend to be cytoprotective. No increase in toxicity could be detected as shown in Fig. 7.

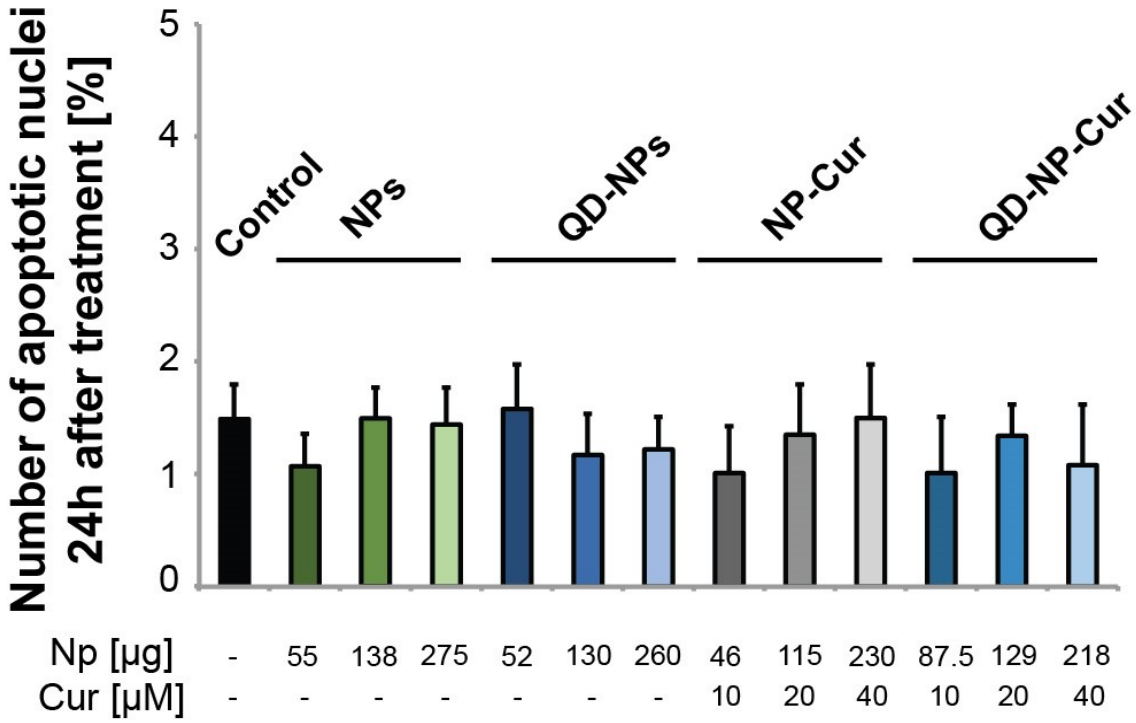


Figure 7: The incubation of Nanoparticles on primary hippocampal rat neurons does not cause apoptosis over 24 hours. After 13 days *in vitro* cells were incubated with three different amounts of Nanoparticles ranging from 46 to 275 µg for 24 hours. The settings were chosen to result in Curcumin concentrations of 10, 20 and 40 µM. Empty and Quantum Dot modified NPs were used in similar concentrations like the Curcumin loaded ones. DAPI staining was used to identify apoptotic nuclei. 10 optical fields were investigated for each condition, n=10. There was no significant difference in the percentage of apoptotic nuclei. Derived from (Barbara et al., 2017).

To investigate if the toxicity varies over time the experiment was repeated for 1 hour, 24 hours and 7 days, as seen in Fig. 8. NPs resulting in a concentration of 20 µM of Curcumin were tested. Even though this is a double dose with 10 µM of Curcumin being recommended, there was no significant change in the rate of apoptosis comparing several incubation times and NP specimen. The Curcumin release profile as shown in Fig. 6 suggests that most of the load has been released after 3 days. This way the 7-day treatment partially includes the disaggregation of the PLGA matrix. We conclude that neither the Curcumin load, nor the PLGA matrix or any of the ligands caused an increase in apoptotic nuclei *in vitro*.

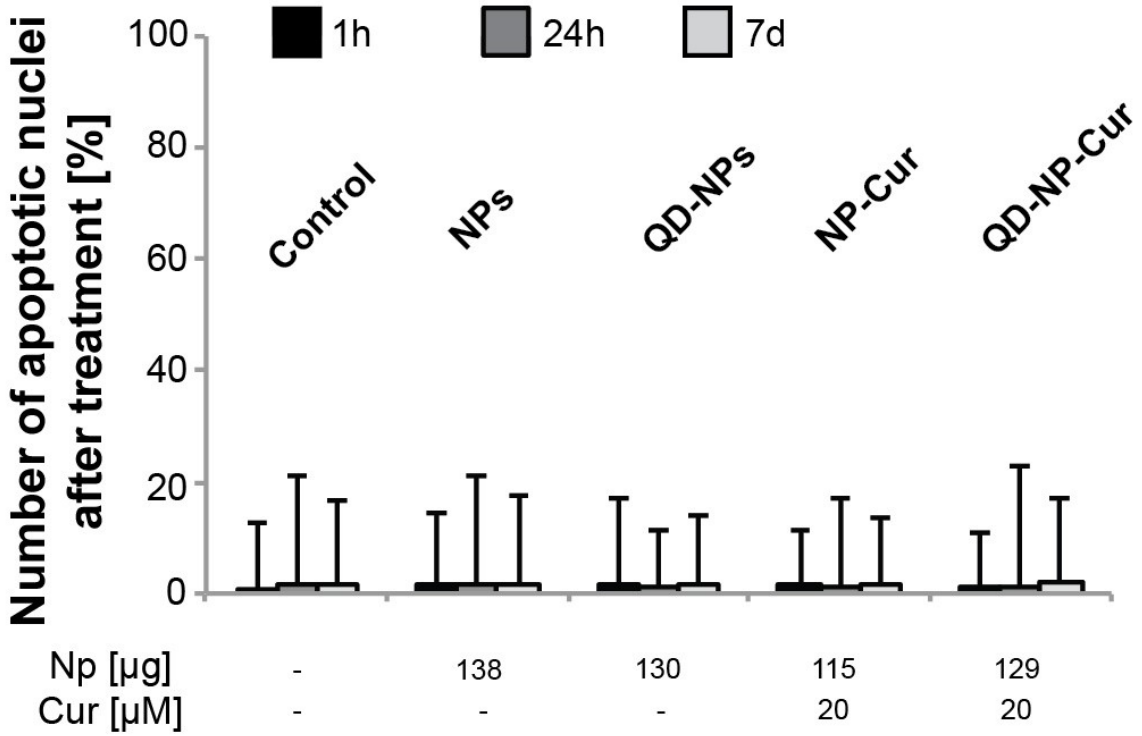


Figure 8: The tested Nanoparticles (NPs) do not cause apoptosis on primary hippocampal rat neurons over 1 hour, 24 hours or 7 days. Loaded NPs resulting in Curcumin concentrations of 20 µM were added. Empty and Quantum Dot modified NPs were used in concentrations comparable to the Curcumin loaded NPs. DAPI staining was used to identify apoptotic nuclei at day *in vitro* 14. 10 optical fields were investigated for each condition, n=10. There was no significant difference in the percentage of apoptotic nuclei. Derived from (Barbara et al., 2017).

The two experiments showed that the NP formulations caused no significant increase in apoptosis. Using four times the recommended Curcumin concentration did not result in toxic effects. Ranging the duration of incubation between 1 hour, 24 hours and 7 days had no effect. There were no differences between the individual NP formulations. This indicates the safety of all the components like the PLGA, Quantum Dots, g7, the polyvinyl alcohol and the Curcumin.

3.3.2. The NPs do not induce necrosis *in vitro*

To evaluate necrotic cell death a healthy/necrotic/apoptotic cell detection kit was used. Empty NPs and NPs containing Curcumin, QDs or both were tested. The Curcumin concentration in the cell culture was set to be 20 µM. Ethidium homodimer III staining showed necrotic cells, while the total number of cells was counted using Hoechst 33342 labeling. 70% ethanol was used for the positive control. Fig. 9 indicates no significant changes in the rate of necrosis after NP incubation for 24 hours.

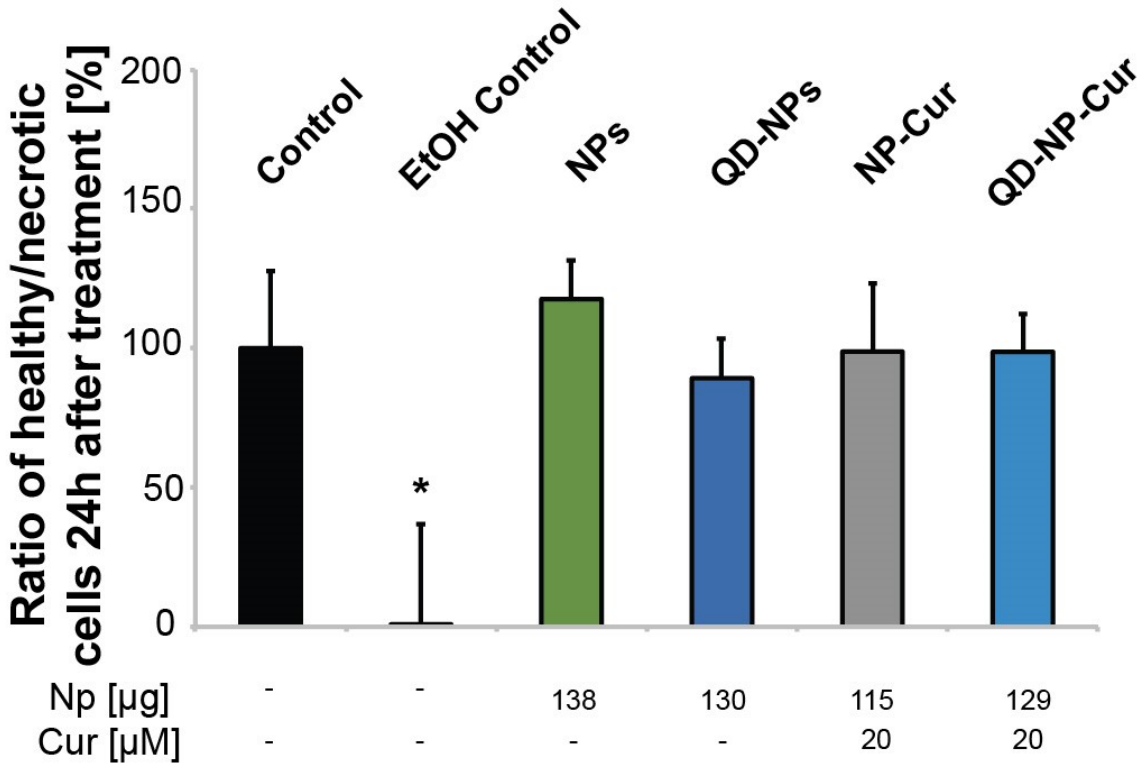


Figure 9: The tested Nanoparticles (NPs) do not cause necrosis on primary hippocampal rat neurons over 24 hours. Loaded NPs were chosen to result in Curcumin concentrations of 20 µM. Empty and Quantum Dot modified NPs were used in similar amounts to the Curcumin loaded ones. After 14 days *in vitro* a healthy/apoptotic/necrotic cell detection kit was used to evaluate toxic effects. Results were normalized against the untreated control. Cells treated with 70% ethanol were used as positive control that showed a significant reduction in healthy cells. 10 optical fields were investigated for each condition, n=10. There was no significant change in the rate of necrosis in the groups incubated with the different NPs. Derived from (Barbara et al., 2017).

We repeated the healthy/necrotic/apoptotic assay with the same amounts of NPs but with an incubation time of 7 days, as demonstrated in Fig. 10. The longer incubation time includes the beginning disaggregation of the NPs and could possibly show toxic effects mediated by its individual components. 70% ethanol treatment was used as a positive control. There were no significant changes in necrosis rates after NP incubation for 7 days *in vitro*.

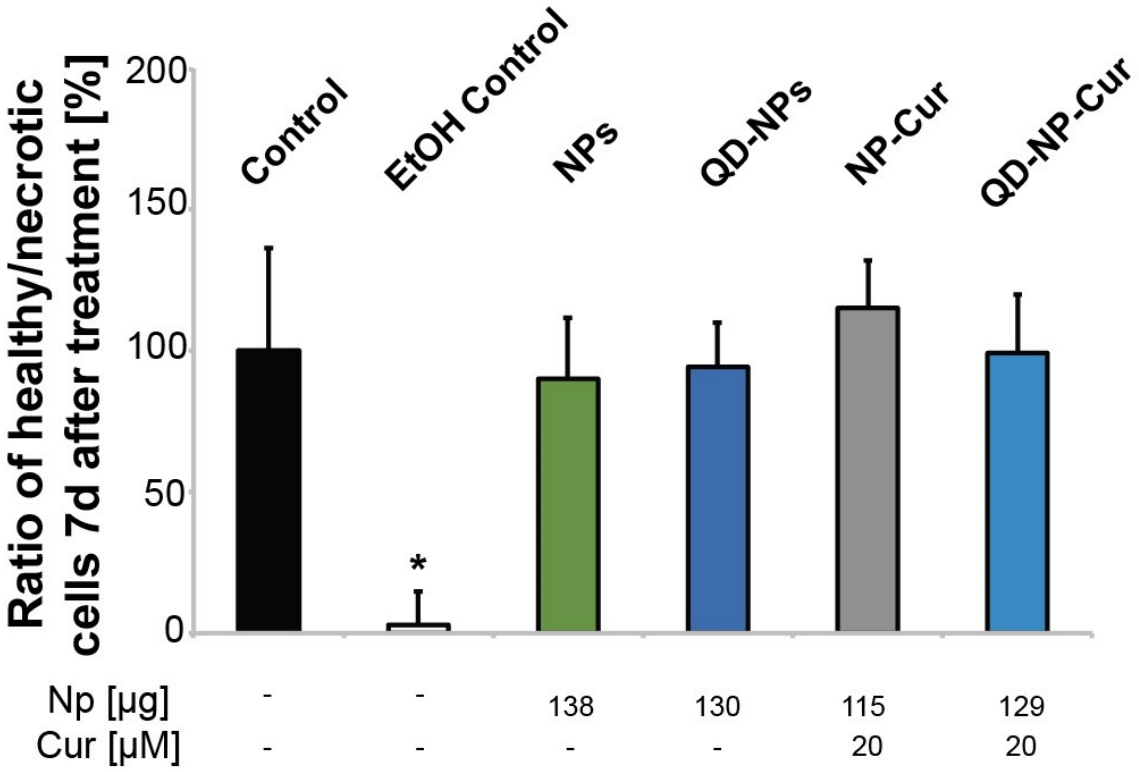


Figure 10: Nanoparticles (NPs) do not cause necrosis on primary hippocampal rat neurons over 7 days. Loaded NPs were chosen to result in Curcumin concentrations of 20 µM. Empty and Quantum Dot modified NPs of the corresponding concentration were used. After 14 days *in vitro* a healthy/necrotic/apoptotic cell detection kit was used to evaluate toxic effects. Results were normalized against the untreated control. Cells treated with 70% ethanol were used as positive control and expressed a significant decrease in cell viability. 10 optical fields were tested for each condition, n= 10. There were no significant changes regarding necrosis in the groups treated with NPs. Derived from (Barbara et al., 2017).

Necrosis is a sign of irreversible cell damage. Fortunately, no increase in necrotic cells was detected after incubation with the different NPs. There also was no difference between the incubation periods of 1 or 7 days. There was no difference between the different NP groups indicating that Curcumin, PLGA and NP modifications of the investigated concentrations do not induce necrosis *in vitro*.

3.4. Biochemical toxicity assays

To detect possible toxic effects that do not show on a cellular but on a molecular level, Western blots for PARP were performed. PARP the poly (ADP-ribose) polymerase is an important enzyme of cellular repair mechanisms. If PARP detects a single strand break of the DNA it is being activated and builds a poly-ADP chain signaling the need for DNA repair mechanisms. If PARP is not activated it will be cut into cleaved PARP which has no more biological activity. Accordingly, high levels of cleaved PARP are a sign of healthy cells.

3.4.1. The NPs do not cause PARP activation over 24 hours

To investigate whether NPs cause DNA damage, cells were treated with different NPs for one day. Cell lysate was obtained from the different treatment groups and then used for Western blots. For each group, triplets were blotted and then incubated with antibodies for PARP and cleaved PARP. Fig. 11 shows the ratios of the mean integrated density of these antibody signals. Free Curcumin and Curcumin loaded NPs were added to result in 10 µM of Curcumin. Empty, QD or g7 modified NPs were added in corresponding amounts. There were no significant changes of the cleaved PARP/PARP ratios and hence no DNA damage attributable to the NP treatment.

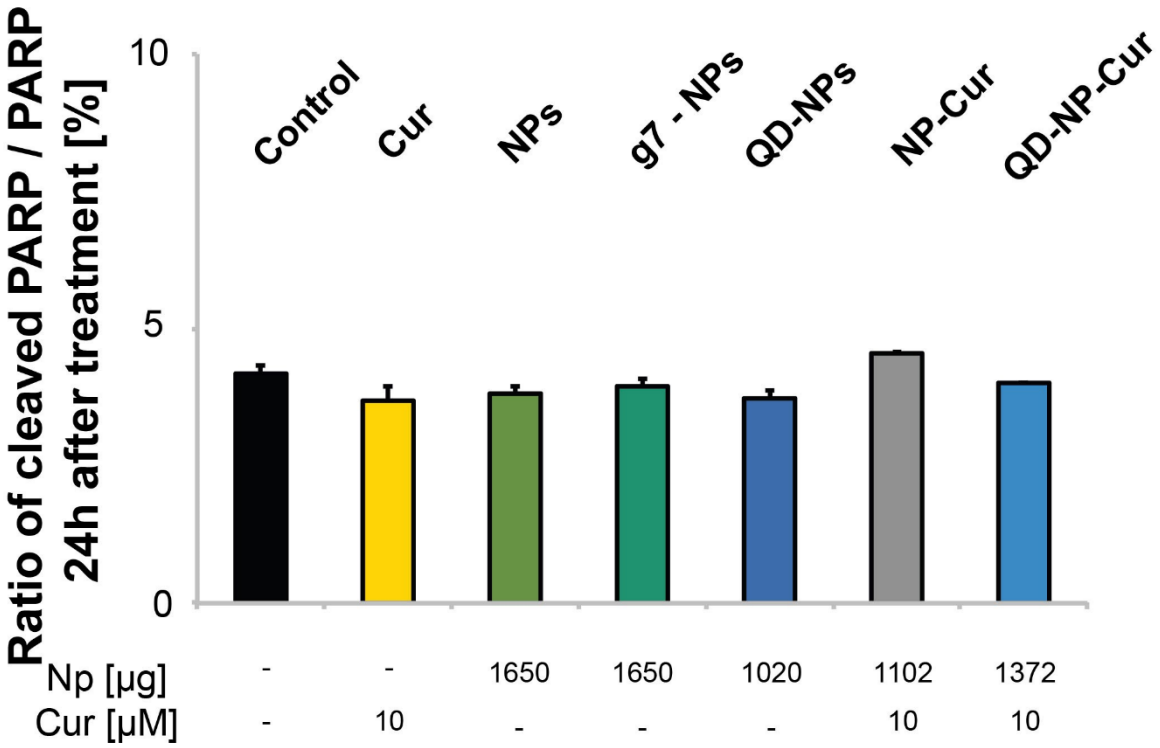


Figure 11: Curcumin and the different tested Nanoparticles (NPs) do not cause activation of the poly (ADP-ribose) polymerase (PARP) on primary hippocampal rat neurons over 24 hours. For 24 hours NPs and free Curcumin of 10 µM were added on petri dishes that had been *in vitro* for 13 days. Empty, Quantum Dot or g7 modified NPs were added in corresponding amounts. A Western blot for PARP and cleaved PARP was used to evaluate molecular cell damage. Triplets were measured for each condition, n=3. There were no significant differences between the groups.

Cell culture treatment with Curcumin or the different NPs for 24 hours does not result in PARP activation. We conclude that there is no molecular cell damage caused by the tested Nanoparticles or any of its compounds at short time.

3.4.2. The NPs do not cause PARP activation over 7 days

The experiment was repeated over 7 days to investigate if the PARP activation is triggered after a longer incubation period. The longer incubation time includes the NP disaggregation which will result in the free circulation of the polymer and the modifications. Fig. 12 shows no sign of DNA damage caused by free Curcumin or NPs with or without encapsulated Curcumin and Quantum Dots.

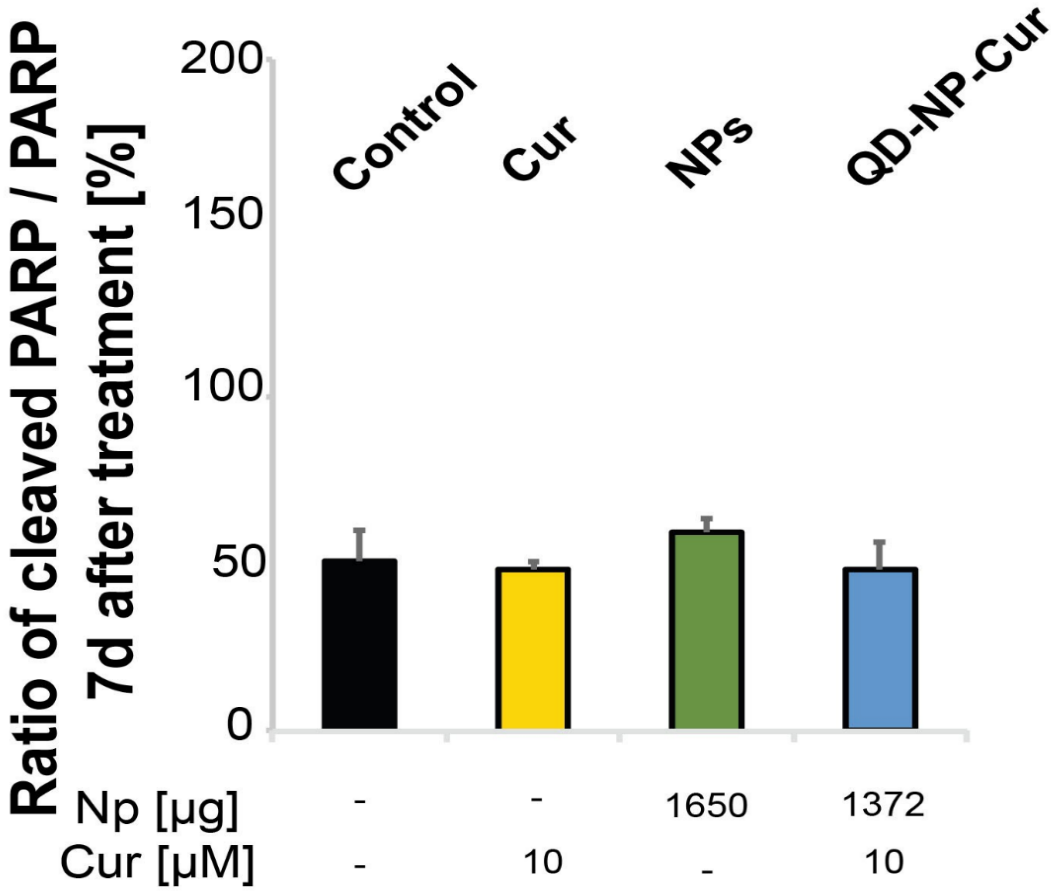


Figure 12: Curcumin, empty Nanoparticles (NPs) and QD-NP-Cur do not cause poly (ADP-ribose) polymerase (PARP) activation on primary hippocampal rat neurons over 7 days. Empty NPs and 10 µM of free Curcumin were used on petri dishes for 7 days. Quantum Dot modified and Curcumin loaded NPs were set to 10 µM of Curcumin. After 14 days *in vitro,* a Western blot for PARP and cleaved PARP was used to evaluate cell damage. Triplets were measured for each condition, n=3. There were no significant differences between the groups.

Neither the free Curcumin nor the different NP formulations resulted in an increase of PARP activation. We conclude that the tested compounds do not result in cell damage on a molecular level when incubated for 1 or 7 days *in vitro*.

For up to 7 days there was no increase in apoptosis, necrosis or PARP activation caused by the different NPs. We conclude that the tested NPs and their individual components do not result in toxicity. Even higher concentrations of Curcumin or Nanoparticles did not result in reduced cell viability.

3.5. Curcumin toxicity

The literature states the effective Curcumin concertation for different applications to be around 10 µM. For Fig. 13 a healthy/necrotic/apoptotic cell detection kit was used to identify toxic effects of Curcumin after 1 and 7 days *in vitro*. Healthy and necrotic nuclei where counted using ImageJ. 70% ethanol was used to obtain a positive control. Curcumin toxicity and cell viability assays showed that a 10 µM solution of Curcumin does not cause significant toxicity. The 10 µM concentration was used for all further experiments except for the Dot Blot. The effects seen at very high Curcumin concentrations are more likely to be caused by the higher amount of the solvent DMSO (Dimethyl sulfoxide) which is known to have toxic effects.

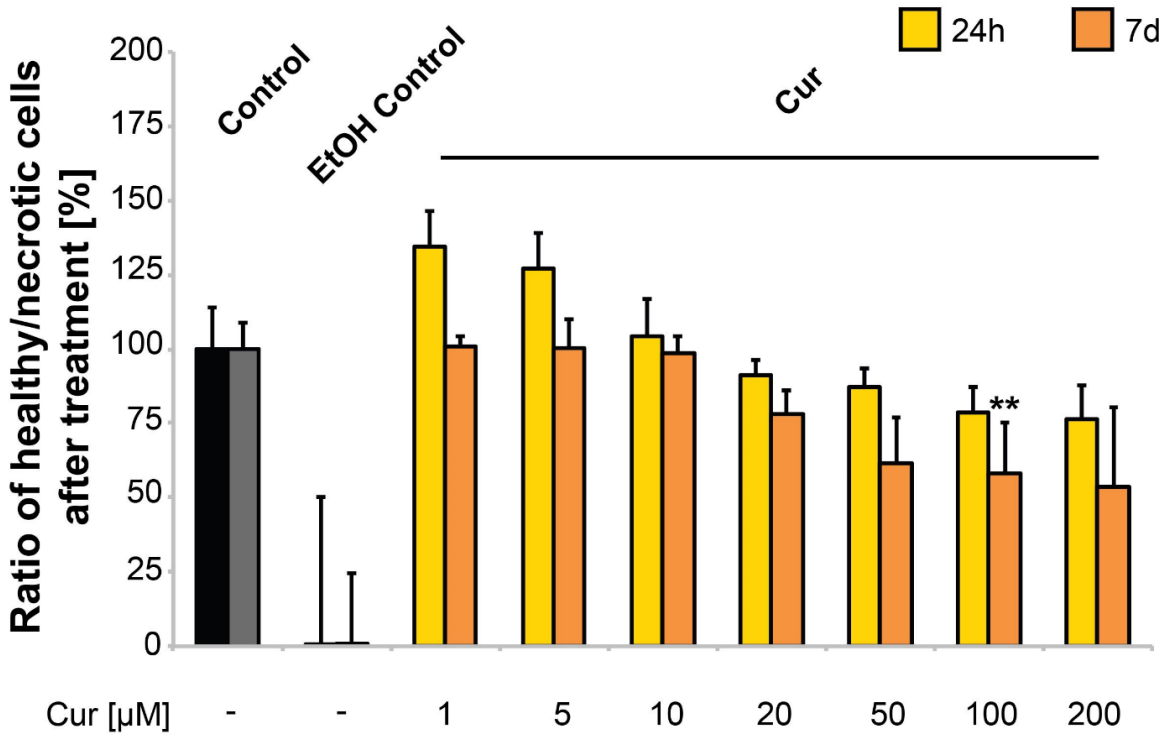


Figure 13: Moderate concentrations of Curcumin do not cause necrosis on primary hippocampal rat neurons over 1 or 7 days. Cells were incubated with different concentrations of Curcumin diluted in Dimethyl sulfoxide (DMSO). The toxicity after overall 14 days *in vitro* was evaluated by the ratio of healthy and necrotic nuclei using a healthy/necrotic cell detection kit and ImageJ. 70% ethanol treatment was used on the positive control. All analyses were performed by investigating 10 optic fields, n=10. Permission for reuse kindly granted by Elsevier. Adapted from (Barbara et al., 2017).

We conclude that a treatment with moderate concentrations of Curcumin for up to 7 days does not have toxic effects on hippocampal neurons *in vitro*. Low doses showed a trend to be cytoprotective. The toxicity caused in higher doses is most likely to be accounted to the DMSO solvent.

3.6. Amyloid beta toxicity

To induce the AD pathology, the cells were treated with 1, 10 and 100 µM of monomeric Aβ for 24 hours, as represented in Fig. 14. 10 µM of Aβ showed a significant reduction in cell viability which was measured by counting apoptotic nuclei in DAPI staining using ImageJ. The concentration of 10 µM was used for all further experiments. The Aβ monomers were always introduced to the cell culture 24 hours before the NPs were added. This way, the monomers could build oligomers and beginning aggregates to induce the AD pathology.

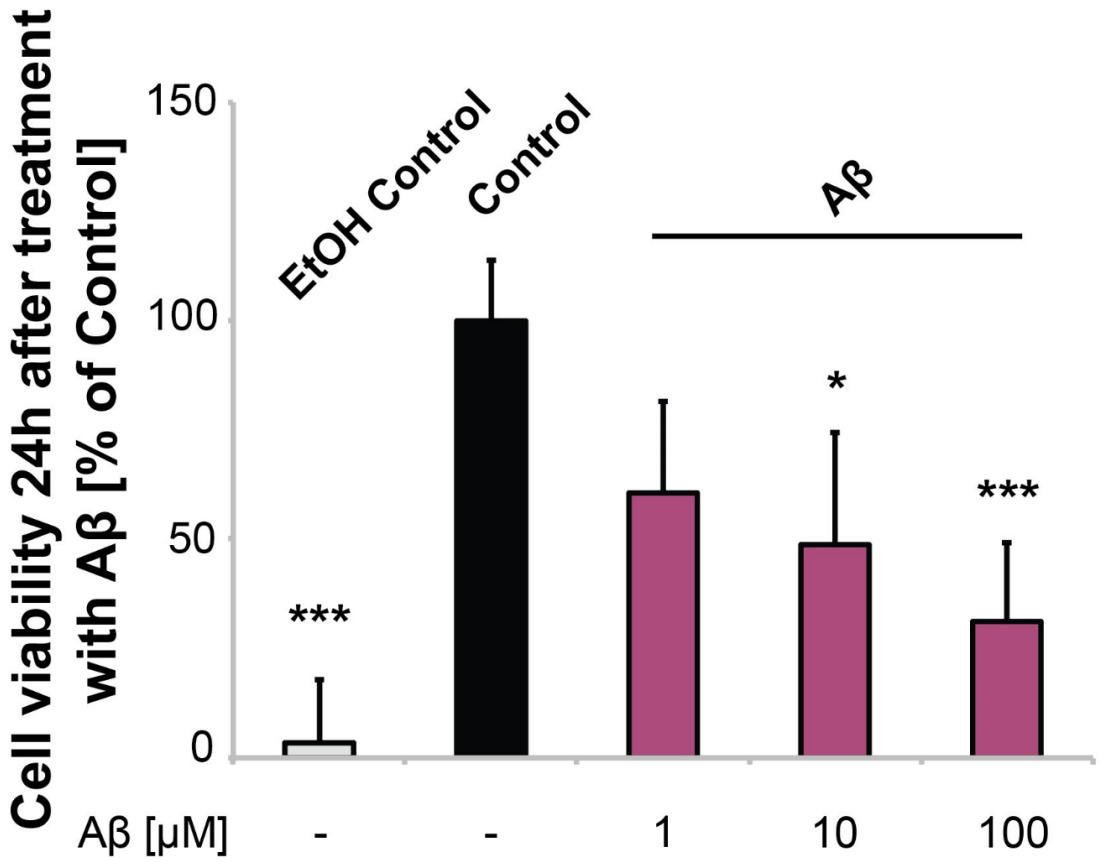


Figure 14: Monomeric Amyloid beta (Aβ) of 10 and 100 µM causes significant reduction in cell viability on primary hippocampal rat neurons over 24 hours. After 13 days *in vitro* cells were incubated with 1, 10 and 100 µM of monomeric Aβ peptide. The toxicity was evaluated by the number of apoptotic nuclei in DAPI staining. 70% ethanol was used for a positive control. All values were normalized to the untreated control. (one-way ANOVA: $F = 6.139$, $p < 0.001$; Bonferroni post hoc test revealed significant differences between control vs. EtOH control $p = 0.003$; control vs. Aβ10 µM $p = 0.07$; control vs. Aβ100 µM $p = 0.0002$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

We conclude that the pretreatment of cells with at least 10 µM of monomeric Aβ for 24 hours is sufficient to introduce AD like toxicity *in vitro.* This allows testing for a possible rescue when NPs are added. The monomers had partially formed aggregates which enables to test Curcumin's plaque disrupting as well as aggregation inhibiting properties.

3.7. Cellular uptake

The fate of NPs after their administration is crucial to estimate their effectiveness. CNS targeted drug loaded NPs should show affinity towards neurons. Cellular uptake is important to cross the BBB (blood-brain barrier) to penetrate the CNS.

3.7.1. Clathrin dependent endocytosis

Cellular uptake and transport mechanisms have great influence on the destination of NPs. Cells were incubated with QD-NP-Cur for 3 hours then fixed and stained for clathrin, to quantify the cellular uptake and the affinity towards neurons. Curcumin has a broad emission spectrum which could interfere with the other signals in the immunocytochemistry. To prevent this interference the QD (Quantum Dot) signal was used to localize the NPs. QDs are strong fluorochromes with a unique and defined signal. Clathrin is a protein of cellular uptake by forming clathrin-coated vesicles. 20 µg of QD-NP-Cur were incubated on primary hippocampal rat neurons at DIV 13 for 3 hours. The merge of the clathrin and the QD signal in Fig. 15 showed a cellular uptake of the QD-NP-Cur by clathrin-dependent endocytosis of 75% within 3 h.

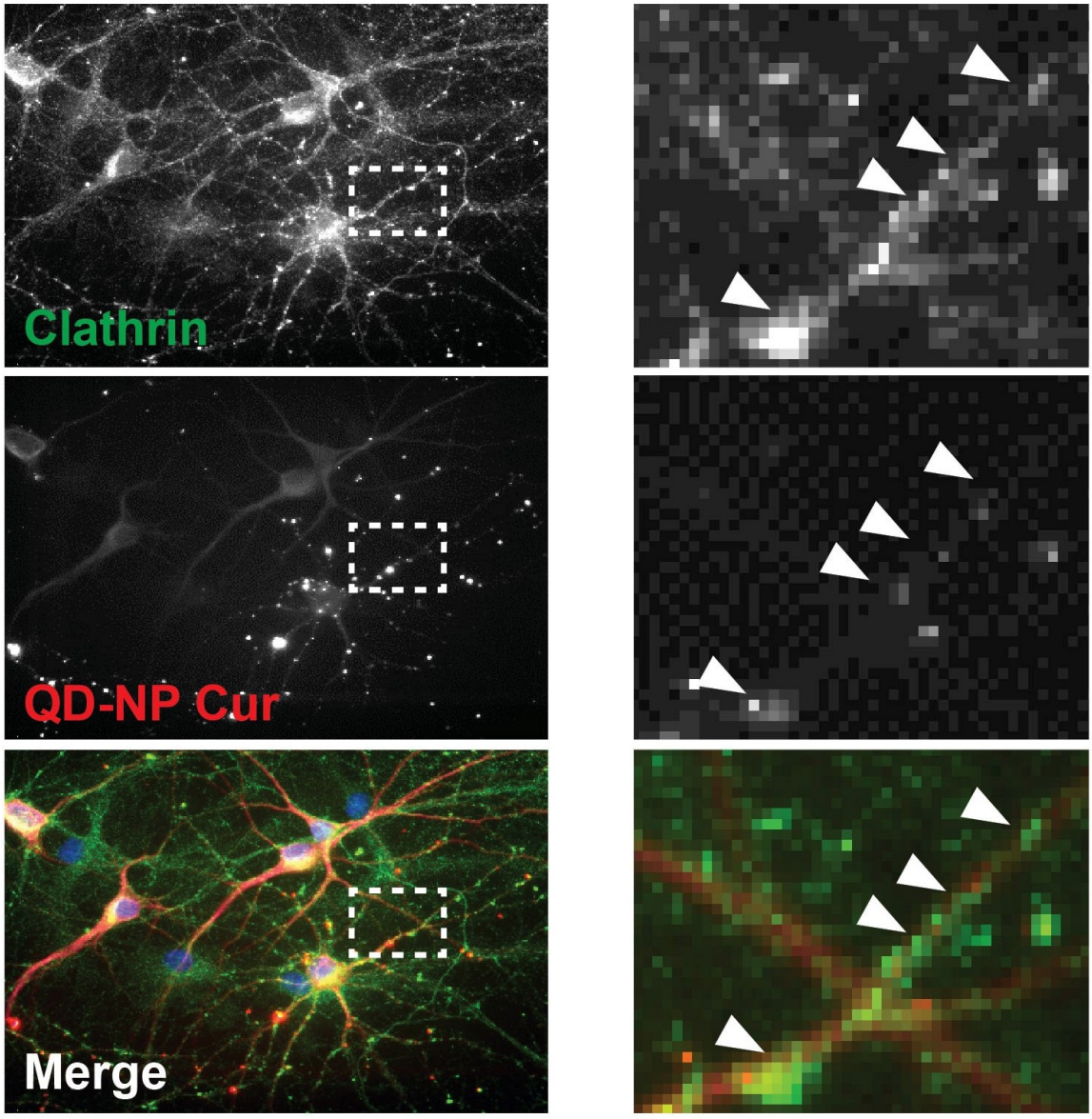
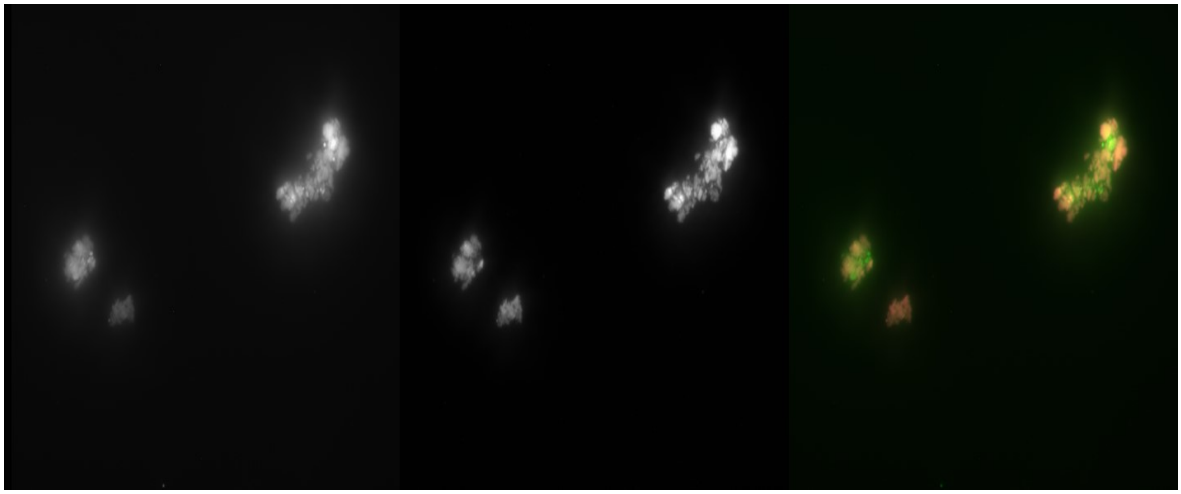


Figure 15: 75% of the Quantum Dot modified and Curcumin loaded Nanoparticles (QD-NP-Cur) are taken up into primary hippocampal rat neurons when incubated with 20 µg for 3 hours. After overall 14 days *in vitro* cells on 24 well plates were fixed and stained for clathrin to show Nanoparticle uptake by clathrin-dependent endocytosis. The co-localization of clathrin and Quantum Dots shows cellular uptake of 75% and affinity of QD-NP-Cur towards neurons.

The results show that the QD-NP-Cur are actively taken up by neurons via clathrin-dependent endocytosis *in vitro*. The images show how defined the QD signal is and how it can be used to localize NPs. The neuronal uptake of the NPs indicates their ability to pass the BBB enabling the treatment of neurological diseases like Alzheimer's disease.

3.7.2. Lysosomal uptake

After cellular uptake, the experiment was repeated using Lamp 2 antibodies to characterize lysosomal uptake. Enrichment of Cy5 inside the lysosomes could be detected. The Cy5 dye is one of the dyes used to visualize the PLGA matrix of the NPs. The fluorescence microscopical images in Fig. 16 show how g7-NP-Cur is actively taken up into neurons and their lysosomes.



Lamp 2 **Cyanin-5-amine** **Merge**

Figure 16: g7-NP-Cur are taken up into lysosomes of primary hippocampal rat neurons when incubated with 20 µg for 3 hours. After overall 14 days *in vitro* cells were fixed and stained for Lamp 2 to show lysosomal co-localization with the Cyanin-5-amine (Cy5) dye.

We conclude that the g7 peptide shows affinity towards neurons and results in a significant cellular uptake of the tested NPs. We observed uptake into primary hippocampal rat neurons via clathrin-dependent endocytosis as well as lysosomal uptake visualized with Lamp 2. This indicates the NPs ability to pass the BBB to enrich within the CNS (central nervous system).

3.8. Aβ aggregation assays

The pathological accumulation of Aβ has been shown to be one of the main causes of cell death in AD. The formed Aβ plaques induce neuroinflammation which results in oxidative stress damaging cells. The inhibition of Aβ aggregation and the disruption of existing plaques are highly desired goals. In Fig. 14 it has been shown that the incubation with 10 µM of monomeric Aβ for one day results in significant toxicity. This pretreatment will be followed by treatment with the different NPs to characterize possible rescue effects.

3.8.1. Western blot for higher order Aβ aggregates

The aggregation of soluble Aβ peptides is known to be the main reason for AD related inflammation and cell health. The disruption of existing plaques or the inhibition of further aggregation could be powerful methods to prevent disease progression. A higher order Aβ antibody was used to differentiate between the Aβ monomers and the bigger Aβ aggregates.

Primary hippocampal rat neurons were preincubated with 10 µM of monomeric Aβ for 1 day to induce the AD pathology. In Fig. 14 it can be seen how this pretreatment results in a significant reduction of cell viability. For Fig. 17 we added 10 µM of Curcumin and NPs resulting in a 10 µM Curcumin concentration to test for a possible rescue over 7 days. At DIV 14 cell lysis was prepared from the different groups and used for a Western blot. The blot was stained for higher order Aβ aggregates to test if Curcumin can reduce the aggregation rate of Aβ.

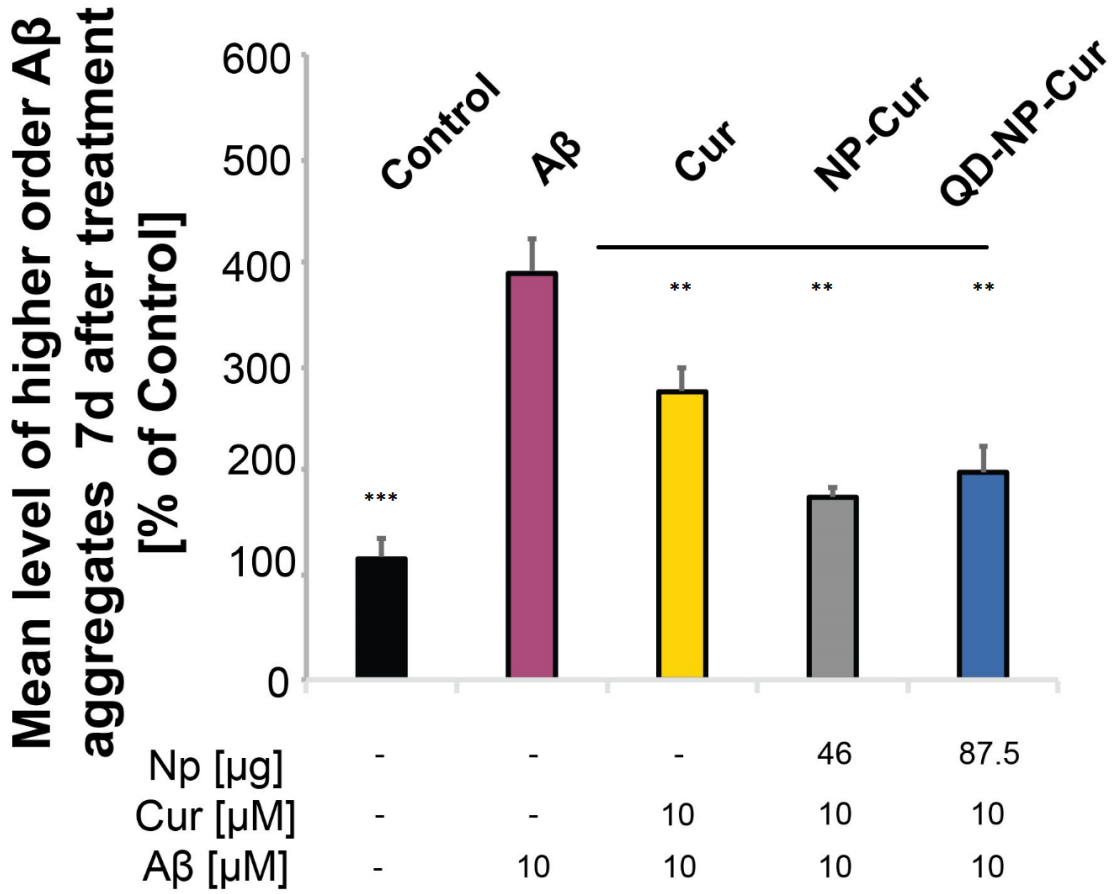


Figure 17: Curcumin loaded Nanoparticles can reduce the built up of higher order Amyloid beta (Aβ) aggregates. Primary hippocampal rat neurons were preincubated with 10 µM of monomeric Amyloid beta for 1 day. Then NPs were added for 7 days resulting in a 10 µM Curcumin concentration. After 14 days *in vitro,* cells were lysed and used in a Western blot to identify higher order Aβ aggregates. Treatment with Cur, NP-Cur and QD-NP-Cur resulted in a significant reduction of higher order Aβ aggregates. Triplets of each group were analyzed, n = 3. (Data was analyzed using one-way ANOVA: f 4-14, F = 4.018, p = 0.034. Letters indicate significant results of Bonferroni post-hoc test from left to right: p = 0.043, p = 0.571, p = 0.163, p = 0.284). Derived from (Barbara et al., 2017).

The incubation with only Aβ monomers resulted in a significant increase of higher order Aβ aggregates. The 7-day treatment with free Curcumin or Curcumin loaded Nanoparticles showed a significant reduction in higher order Aβ aggregates in a Western blot using triplets of every group. We conclude that 10 µM of Curcumin can effectively inhibit Aβ aggregation *in vitro*.

3.8.2. Thioflavin T based Aβ aggregation assay

A Thioflavin T based Aβ aggregation assay was performed using a Microplate reader (Multiskan RC). The aggregation of Aβ42 and Thioflavin results in an emission peak at a wavelength of 484 nm after an excitation with 440 nm. The incubation of just Aβ and Thioflavin in Fig. 18 expressed a strongly significant increase in fluorescence and hence in aggregation. Phenol Red which is a known inhibitor of Aβ aggregation was used as control. Empty NPs increased the Aβ aggregation at short time. This can most likely be attributed to the ability of empty NPs to attract peptides. This results in higher local Aβ concentrations on the NP's surface promoting the aggregation in the beginning. However, this very effect will partially inhibit the formation of higher order Aβ aggregates by binding Aβ fibrils. The Curcumin loaded Nanoparticles demonstrated a strongly significant reduction in fluorescence. NPs were chosen to result in 40 µM of Curcumin. The signal intensity at t = 0 min was defined to be zero. This way the g7-NP-Cur treated group showed negative signal intensity and hence negative Aβ aggregation. Consequently, Curcumin does not only inhibit the aggregation of Aβ but also disrupts existing plaques.

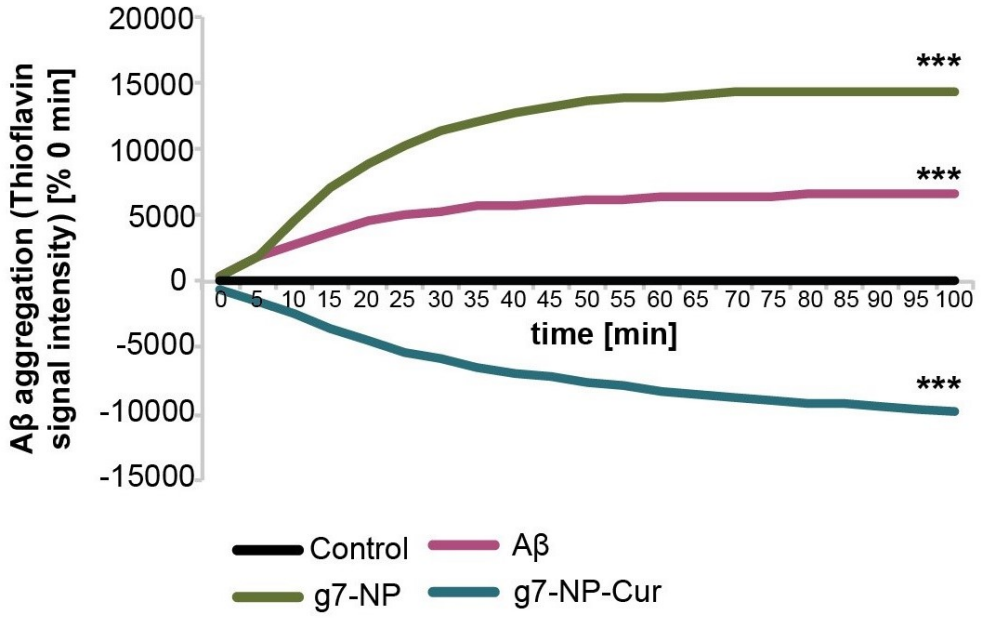


Figure 18: Curcumin loaded Nanoparticles inhibit Amyloid beta (Aβ) aggregation and mediate Aβ disaggregation. A SensoLyte® Thioflavin T beta-Amyloid (1-42) Aggregation Kit was performed using a Microplate reader measuring the relative fluorescence units over time. Aβ42 and Thioflavin aggregation results in an increased signal intensity indicating Aβ aggregation. Phenol Red as known aggregation inhibitor was used as control. Treatment with only Aβ resulted in a strongly significant increase in signal intensity. Likewise, did the additional treatment with empty g7-NP. The treatment with g7-NP-Cur in contrast resulted in a strongly significant decrease in signal intensity indicating Aβ disaggregation. The Curcumin concentration has been set to 40 µM. (one-way ANOVA, F: 166.839, $p < 0.0001$; Bonferroni post hoc test revealed strongly significant differences between all groups $p < 0.001$.) Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

We conclude that Curcumin can inhibit Aβ aggregation and mediate plaque disrupting in a physiological medium. This suggests Curcumin's ability to inhibit the AD progression by a reduction of the existing plaque burden and inhibition of new plaque buildup. This is especially promising, because it suggests that Curcumin can not only slow down disease progression but can also mediate the reduction of existing plaques.

3.8.3. Dot Blot for original conformation Aβ

A Dot blot analysis was performed detecting the native and original conformation (OC) of Aβ with an OC Aβ antibody. The OC Aβ antibody is able to recognize fibrillary oligomers that are immunologically distinct from prefibrillar oligomers. Primary hippocampal rat neurons were grown on petri dishes and pretreated with 10 μM of monomeric Aβ. 24 hours later different groups were treated with free Curcumin, g7-NP, g7-NP-Cur or left as control. Supernatants of these cell cultures were collected after 7 days of incubation at DIV 14. A Dot Blot of the ultra-centrifuged supernatants from the different treatment groups was prepared and stained for OC Aβ. There was a strongly significant increase in OC Aβ levels in the groups treated with Aβ peptide compared to the control. The Curcumin treated group had a significant decrease in OC Aβ and the Curcumin loaded Nanoparticles even a strongly significant one compared to the positive control treated with only Aβ. This indicates an advantage of the Nanoparticle formulation over the free Curcumin. The sustained release from the Nanoparticles maintains an effective dosage of Curcumin over a longer period. The triplet group treated with empty g7-NP showed no effect, suggesting that the positive effects in other treatment groups were achieved by Curcumin. Fig. 19 shows both the Dot blot and its results.

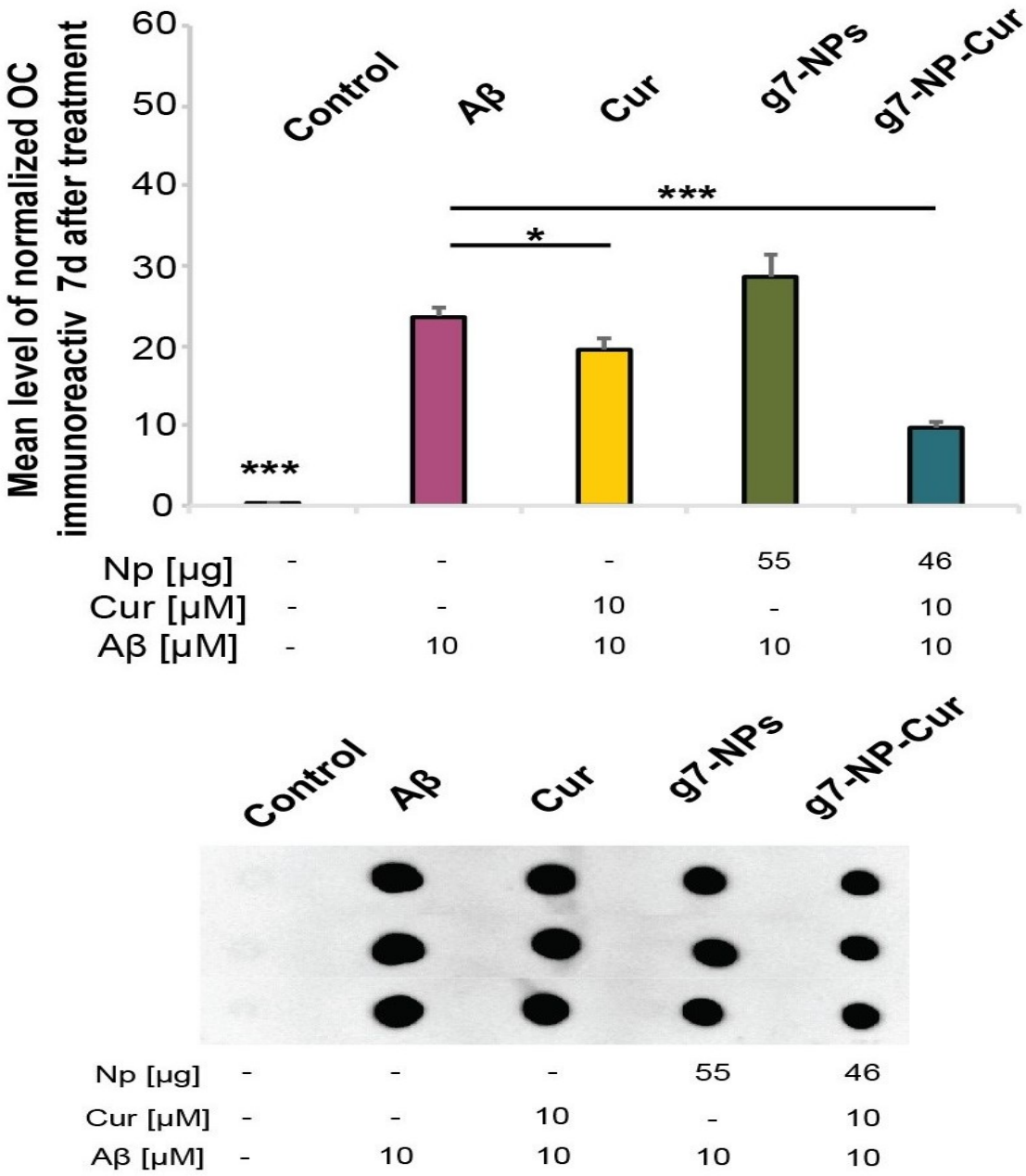


Figure 19: Curcumin and NP-Cur reduce the concentration of the original conformation (OC) Amyloid beta (Aβ) on primary hippocampal rat neurons over 7 days. Cells were grown on a petri dish and pretreated with 10 µM of monomeric Amyloid beta for one day. Free Curcumin, g7-NP and g7-NP-Cur were added for 7 days. The Curcumin concentration was set to 10 µM. Supernatant was obtained from the groups after 14 days *in vitro.* Supernatants were ultra-centrifuged and doted on a nitrocellulose membrane. The OC Aβ antibody was used to quantify the level of OC Aβ in the groups. The signals were normalized against the signal of Ponceau S of the corresponding dot representing the overall protein content. Treatment with free Curcumin results in a significant reduction of OC Aβ. G7-NP-Cur treatment results in a strongly significant reduction of OC Aβ (one-way ANOVA: $F = 91.639$, Aβ and Aβ + Cur $p = 0.049$, Aβ and Aβ + g7-NP-Cur $p < 0.001$. Bonferroni post hoc test revealed significant differences between all groups and the Control $p < 0.001$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

The results show that the aggregation of the original conformation of Aβ can indeed be inhibited by Curcumin. For the 7-day incubation time the Curcumin loaded NPs are more effective than the free Curcumin indicating an advantage of the sustained release.
The Western blot, the Dot Blot and the Thioflavin T based Aβ aggregation assay all show Curcumin to significantly reduce Aβ aggregation.

3.9. Reduction of overall plaque count

It was investigated whether the Curcumin and Curcumin loaded NPs can reduce the overall number of plaques *in vitro*. For Fig. 20 the concentrations of 10 µM of monomeric Aβ and 10 µM of Curcumin were maintained. Primary hippocampal rat neurons were pretreated with monomeric Aβ for one day. Then the different specimens of Nanoparticles were added for 7 days. The Aβ antibody from Sigma-Aldrich and a Synaptic Systems MAP 2 antibody were used to visualize both the neurons and the Aβ plaques by immunocytochemistry. The neurons are visible in red while the Aβ plaques can be seen in green. Per condition, 10 optical fields were measured using the ImageJ software. There was a strongly significant reduction in the number of plaques in the groups treated with Curcumin or Curcumin loaded Nanoparticles. In Fig. 18 the Thioflavin T based Aβ aggregation assay showed a trend that empty NPs could promote Aβ aggregation at very short time because the NP's surface is able to attract peptide. Here we can see that this effect can no longer to be measured after 7 days. There is even a trend towards a smaller plaque number after treatment with empty NPs.

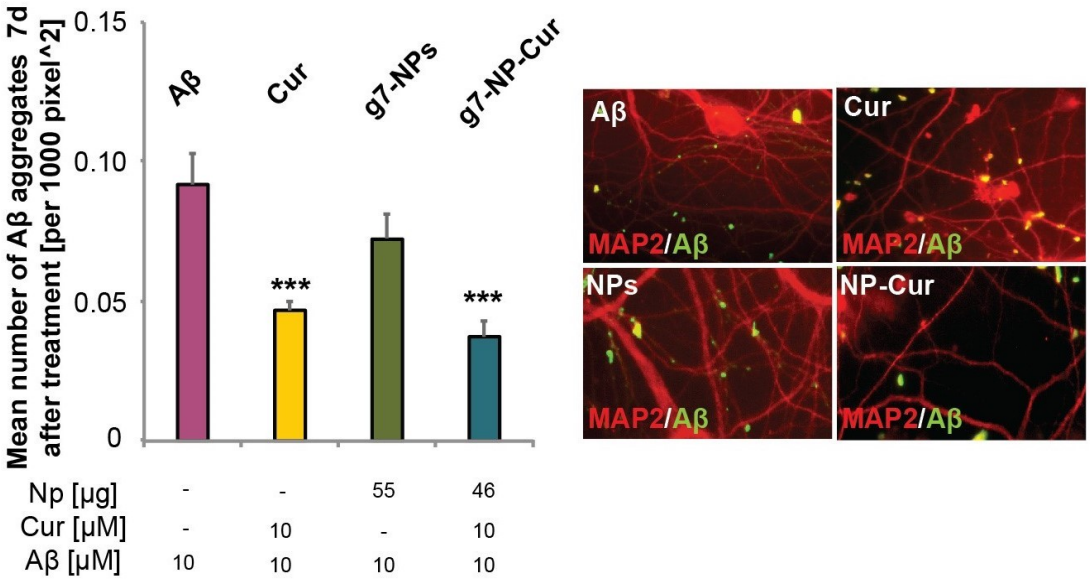


Figure 20: The number of Amyloid beta (Aβ) plaques on primary hippocampal rat neurons decreases after treatment with Curcumin or g7-NP-Cur for 7 days. Cells were pretreated with 10 µM of monomeric Aβ for 1 day. Then the Nanoparticles or Curcumin were added for 7 days. After 14 days *in vitro*, cells were stained for Map2 and Aβ using antibodies. There was a strongly significant reduction in plaque number for cells treated with Cur or g7-NP-Cur (one-way ANOVA: $F = 34.493$, $p < 0.0001$; Bonferroni post hoc test revealed significant differences between Aβ and Aβ + Cur $p < 0.001$, and Aβ + g7-NP-Cur $p < 0.001$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

The results confirm the aggregation assays and indicate that Curcumin and Curcumin loaded NPs can significantly reduce the overall number of Aβ plaques *in vitro*.

3.10. Cell viability after Aβ and NP treatment

To investigate if the reduction of Aβ aggregates has effects on the overall cell survival another cell viability assay was performed using the healthy/necrotic/apoptotic cell detection kit, as shown in Fig. 21. Again, cells were pretreated with 10 µM of Aβ for one day. Then, the NP specimens were added to result in 10 µM of Curcumin and left to incubate for 7 days until DIV 14. 70% ethanol was used as positive control and showed significant reduction in cell viability in this group. There is a clear trend towards a rescue comparing the groups treated with only Aβ with the groups that were also treated with Curcumin or NP-Cur.

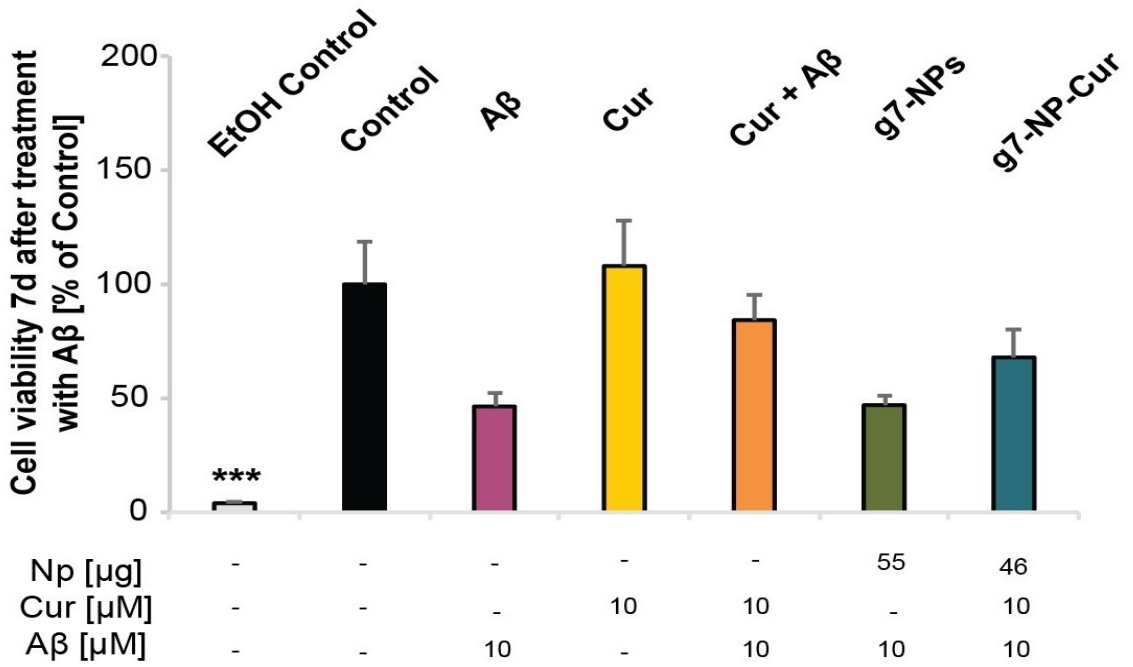


Figure 21: Curcumin and Curcumin loaded Nanoparticles (NPs) show a trend to mediate a rescue in primary hippocampal rat neurons pretreated with Amyloid beta (Aβ). Cells were pretreated with 10 µM of monomeric Aβ for one day. Free Curcumin, g7-NP and g7-NP-Cur were added to incubate for 7 days. The Curcumin concentration was set to be 10 µM. After 14 days *in vitro* cell viability was evaluated by apoptotic nuclei in DAPI staining. 70% ethanol was used as positive control (one-way ANOVA: $F = 43.316$, $p < 0.001$; Bonferroni post hoc test revealed significant differences between all conditions and the EtOH control $p < 0.001$). Permission for reuse kindly granted by Elsevier. Adapted from (Barbara et al., 2017).

There is a trend that Curcumin can improve overall cell survival after induction of the AD pathology. Even though not significant the results are still promising since the incubation time of only 7 days is a relatively short time span compared to the years of Aβ accumulation in AD.

3.11. Synapse count

Synapse count is a good cellular correlate to evaluate the cognitive impairment in AD. It has been shown that Aβ causes synapse loss in hippocampal cells. Synapse density and number are correlating with overall brain health and it has been shown that there is a reduction of synapse density in AD. For this reason, a rescue experiment was performed in which cell cultures were treated and later stained for the synaptic protein Homer1 to count synapses using a fluorescence microscope and ImageJ. The toxic effects of Aβ caused a strong reduction in synapse density. The treatment with Curcumin loaded Nanoparticles showed a trend towards a rescue effect. The results were not significant but showed a trend. Other groups had shown that Curcumin has a protective effect on synapse health and density.

In the experiment corresponding to Fig. 22 it was evaluated whether Curcumin can alleviate the synapse loss caused by Aβ aggregation. Cells were pretreated with 10 µM of Aβ for one day before being treated with NPs resulting in 10 µM of Curcumin for 7 days. At DIV 14 immunocytochemical staining for Homer1 was used to visualize synaptic densities. ImageJ was used to measure the number of synapses that was then divided by the number of neurons in the optical field. Two trends could be seen. Firstly, Aβ seems to reduce the number of synapses per neuron as stated in the literature. Secondly, it appears that the g7-NP-Cur can partially rescue the cells in its group from loosing as many synapses as the other two Aβ treated groups. It should be taken into consideration that an incubation time of 7 days is short compared to AD which can take decades to reach its typical characteristics.

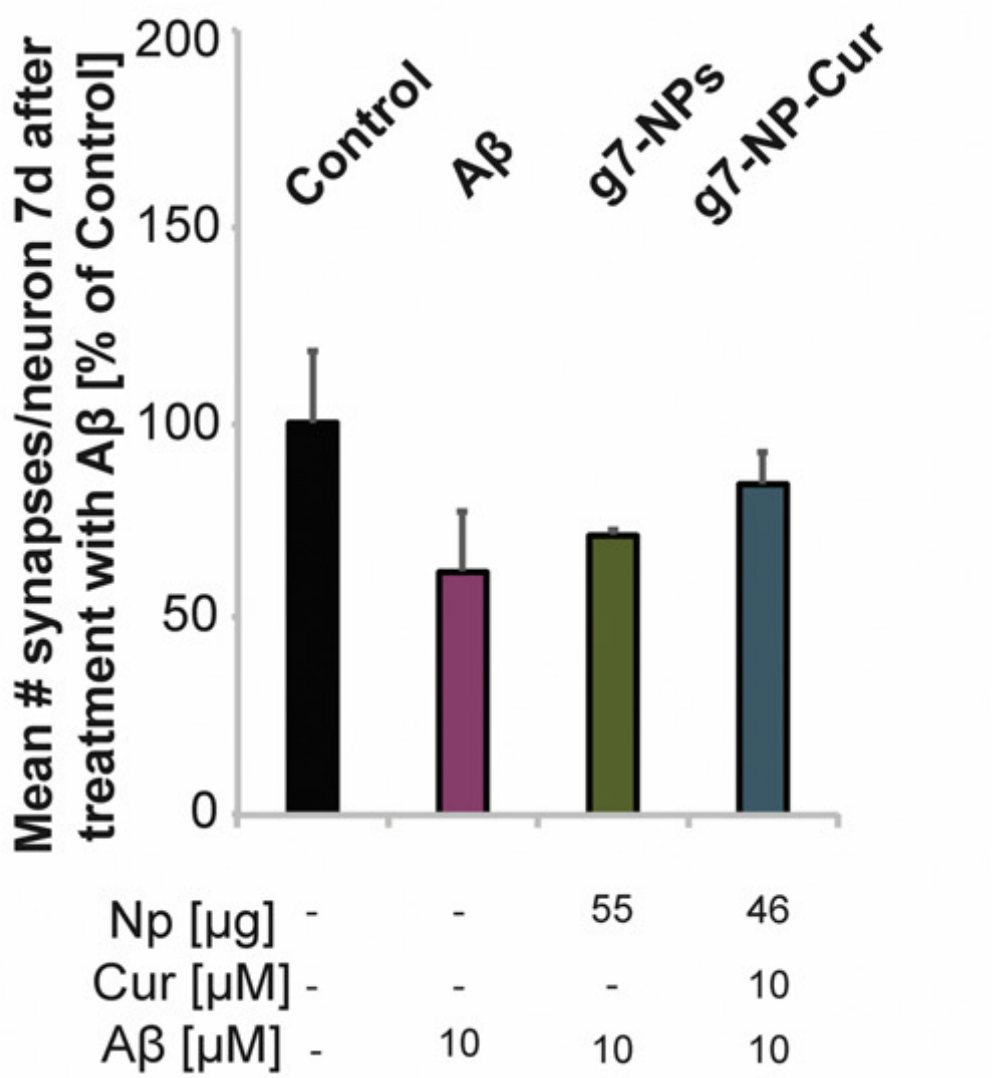


Figure 22: Curcumin loaded Nanoparticles show a trend to rescue cells from Amyloid beta (Aβ) induced synapse loss. The mean number of synapses per neuron was measured after exposing primary hippocampal rat neurons to 10 µM of Aβ for 1 day followed by a 7-day treatment with g7-NP or g7-NP-Cur. The concentration of g7-NP-Cur was chosen to result in 10 µM of Curcumin in the culture medium. Synapses were stained for Homer1 and their number per neuron was measured. There were no significant differences between the groups. Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

The results show a trend of Aβ reducing the synapse density. There is also a trend of Curcumin partially inhibiting this reduction. To receive more significant results this experiment should be repeated using longer incubation times or preferably an *in vivo* study.

3.12. Anti-inflammatory properties

It has been shown that the main reason for cell loss in AD is the inflammatory response that is triggered by the Aβ plaques. Once the Aβ has built plaques it induces microglial activation which initiates inflammatory processes by NF-κB activation. This "nuclear factor kappa-light-chain-enhancer of activated B-cells" plays a key role in the inflammatory cascade. Its inhibition has been shown to reduce inflammatory cytokines and reactive oxygen species. While inflammation is necessary under physiological conditions, it is a major cause for cell damage when chronic inflammation occurs.

NF-κB is activated by the phosphorylation and degradation of the inhibitor of kappa B (IκB). To investigate the anti-inflammatory properties of Curcumin, an antibody detecting unphosphorylated IκB was used to measure inflammation in an immunocytochemical assay. The treatment with g7-NP-Cur showed significant rescue of IκB levels compared to groups treated with only Aβ or g7-NP plus Aβ. IκB in Curcumin treated groups reached a higher level than in the control group without the presence of Aβ. This shows that the anti-inflammatory effect of Curcumin is given without being triggered. This implies the possibility to use Curcumin as prevention or in early stages. The corresponding results are shown in Fig. 23.

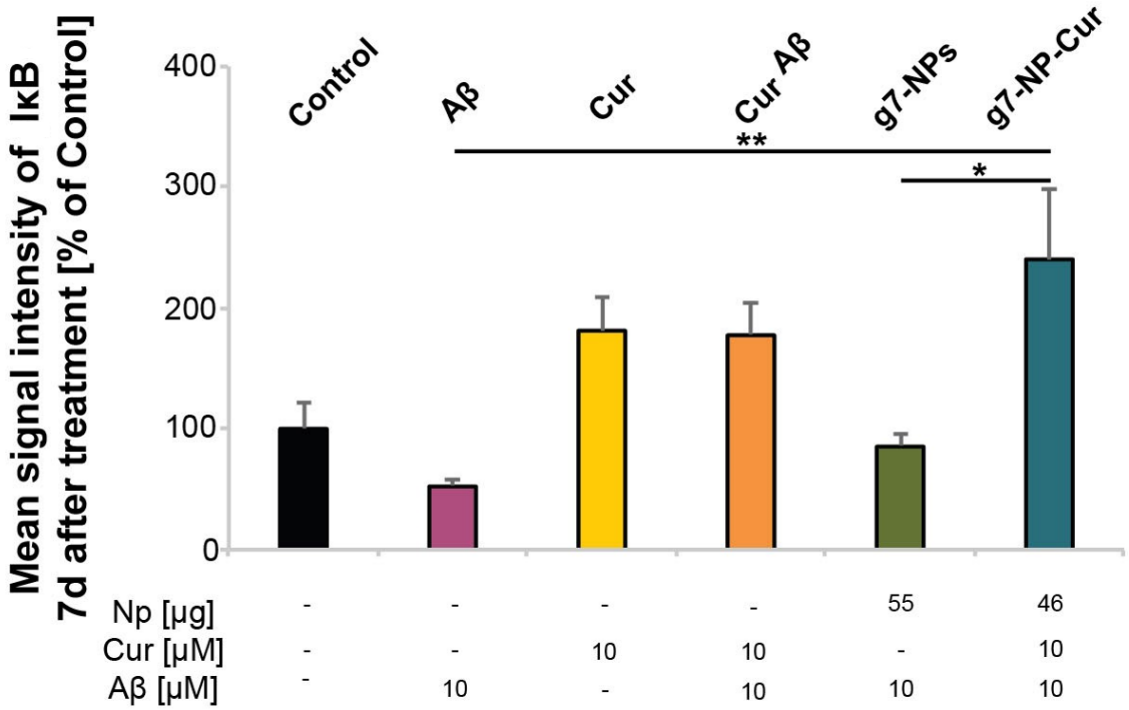


Figure 23: Curcumin and Curcumin loaded Nanoparticles (NPs) can rescue primary hippocampal rat neurons from inflammation caused by Amyloid beta (Aβ) over 7 days. Cells were pretreated with 10 µM of monomeric Aβ for one day. Free Curcumin and NPs resulting in 10 µM of Curcumin were added for 7 days. G7-NP were added in a similar concentration. The inhibitor of κB (IκB) was measured by immunocytochemistry after overall 14 days *in vitro*. Ten optical fields were measured for each group, n = 10. (one-way ANOVA: $F = 4.242$, $p < 0.001$; Bonferroni post hoc test revealed significant differences between Aβ and Aβ plus g7-NP-Cur treated cells $p = 0.002$, and between Aβ plus g7-NP and Aβ plus g7-NP-Cur treated cells $p = 0.021$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

We conclude that Curcumin is able to inhibit Aβ induced neuroinflammation. IκB levels in the Curcumin treated groups were even higher than in the untreated control. This indicates that Curcumins potent anti-inflammatory properties are given even before inflammation is induced. This could have promising effects regarding the prevention of the severe inflammation in early stages of AD.

Since inflammation plays an important role in the pathology of many different frequent diseases like rheumatoid arthritis, inflammatory bowel disease or multiple sclerosis, there is a broad range of possible indications for Curcumin.

3.13. Antioxidant properties

In AD, the pathological aggregation of Aβ causes a chronic inflammatory response. Proinflammatory mediators induce microglial activation and cause them to release reactive oxygen species (ROS). The term ROS includes different highly reactive radicals that can neutralize pathogens under physiological conditions. However, ROS can also damage healthy cells by oxidizing their cell membrane, proteins and DNA. There is a variety of other chronic inflammatory diseases were a reduction of free radicals could reduce cell damage.

Curcumin displays a keto-enol tautomerism meaning that both the keto and the enol conformation can prevail depending on the solvent medium. The electrons inside the phenol rings are delocalized. This enables Curcumin to absorb singled electrons and thereby act as a radical scavenger.

To evaluate the antioxidant properties of Curcumin, oxidative stress had to be introduced to the cell culture using the Fenton's reaction. Cells were preincubated with H_2O_2 and $FeCl_2$ for 24 hours to cause significant oxidative stress. H_2O_2 was used to oxidize the Fe^{2+} that had disassociated of the $FeCl_2$. The H_2O_2 is being reduced by receiving the electron from the Fe^{2+}. In this process the molecule falls apart into a hydroxyl ion and a hydroxyl radical. Radicals are defined by their free unpaired electrons which cause them to be very unstable and reactive. Therefore, radicals can oxidize lipid membranes, proteins and DNA causing severe damage that often leads to cell death.

Fenton's reaction: $\mathbf{Fe^{2+} + 2Cl^- + H_2O_2 \rightarrow Fe^{3+} + 2Cl^- + \cdot OH + OH^-}$

To visualize the effects a CellROX® Green Reagent Kit from Thermo Fisher was used. The positive control in Fig. 24 shows how the Fenton's reaction strongly significantly increased the oxidative stress in the cell culture.

3.13.1. Curcumin as a radical scavenger

Fig. 24 demonstrates how Curcumin can be used for its ability to neutralize ROS as a radical scavenger. The preincubation with the oxidative Fenton's reagents for 1 day significantly increased oxidative stress as detected with the CellRox® kit. Next, 10 µM of free Curcumin were added for 30 minutes or 1 day showing a strongly significant reduction of oxidative stress. The CellRox® kit was used to measure free radicals in a immunocytochemical assay. Curcumin reduced oxidative stress to a lower level than in the untreated control group.

In a fifth group, only Curcumin was added showing that it is able to reduce the oxidative stress even when it is not introduced by the Fenton's reaction. Curcumin treated groups had less ROS than the base level in the control group. This base level represents the oxidative stress caused by background radiation and other sources of ionizing radiation. This contributes to the cytoprotective properties of the polyphenol Curcumin. It also confirms the preventive role of Curcumin and suggests an advantage of beginning treatment in early or preclinical stages.

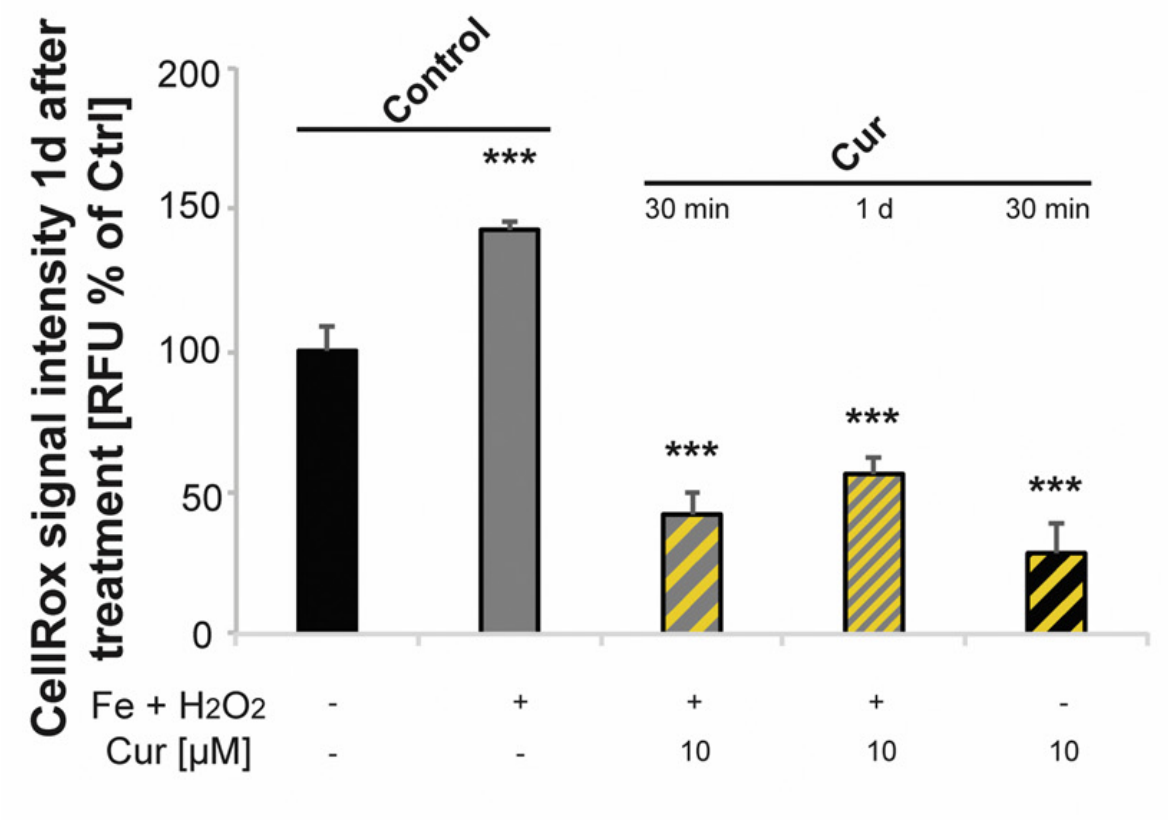


Figure 24: Curcumin can reduce oxidative stress induced by the Fenton's reaction on primary hippocampal rat neurons. The Fenton's reagents (0.5 µM of $FeCl_2$ and 2.5 µM of H_2O_2) had preincubated with the cells on a 24 well plate for 1 day. 10 µM of Curcumin were added for 1 day or 30 minutes the day after the radicals were introduced. A CellRox® oxidative stress detection kit was used to measure free radicals at day *in vitro* 14. The groups treated with Curcumin had significantly lower levels of oxidative stress compared to the positive control and even less than the untreated control. Treatment with Curcumin alone reduced oxidative stress to a level lower than in the untreated control. All values were normalized to the untreated control. (Bonferroni post hoc test revealed strongly significant differences between control and positive control and between control and all Curcumin treated groups (one-way ANOVA: $F = 8.554$, $p < 0.001$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

We conclude that free Curcumin can neutralize oxidative stress over 1 day by acting as a radical scavenger. Even in the group where no oxidative stress was introduced Curcumin reduced the base level of ROS to a degree less than in the untreated group. This shows its potent ability to guard cells from damage caused by free radicals.

3.13.2. Curcumin loaded NPs reduce oxidative stress *in vitro*

To verify longer lasting effects, the second CellRox® experiment for Fig. 25 was performed over 7 days with 1 day of preincubation with the Fenton's reagents. Curcumin loaded and empty NPs were used to evaluate the role of the NPs. The Curcumin concentration was set to 10 µM. Again, the positive control had significantly more free radicals in the culture showing that the radicals can remain within the culture for up to 7 days. The g7-NP-Cur treated group showed a strongly significant reduction of free radicals. The Curcumin loaded NPs reduced ROS to a level lower than the base level of the untreated control even after 7 days. This shows the prolonged effects of Curcumin especially after the sustained release out of the Nanocarriers. Even the empty NPs caused a reduction of ROS. The delivery of Curcumin by g7-NP-Cur can be used to neutralize oxidative stress *in vitro* for up to 7 days.

In AD the ROS are released because of the neuroinflammation that is triggered by the Aβ. We could show that Curcumin and the Curcumin loaded Nanoparticles are able to reduce Aβ aggregation, neuroinflammation and oxidative stress. These are the three main steps leading to neuronal cell loss caused by Aβ. In AD these three pathologies occur simultaneously. Curcumin acts upon all three steps indicating that its combined effects could be potentiated. The disruption of Aβ plaques results in less of an inflammatory response. This reduction of inflammation results in lower levels of ROS which attributes to less cell loss by oxidative stress.

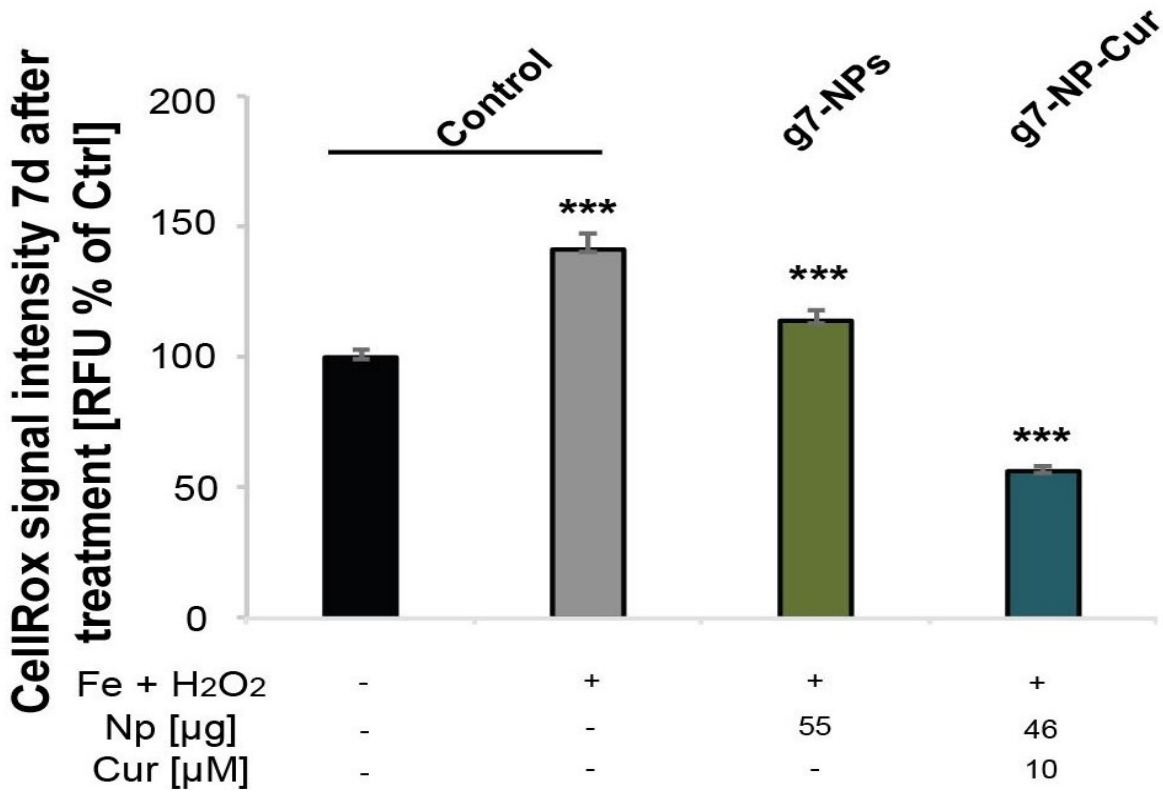


Figure 25: Curcumin loaded Nanoparticles (NPs) reduce oxidative stress induced by the Fenton's reagents on primary hippocampal rat neurons over 7 days. The Fenton's reagents (0.5 µM of $FeCl_2$ and 2.5 µM of H_2O_2) had preincubated with the cells on a 24 well plate for 1 day. Then g7-NP and g7-NP-Cur resulting in 10 µM of Curcumin were added for 7 days. Oxidative stress was measured at the 14. day *in vitro* using the CellRox® oxidative stress detection kit. The positive control showed a strongly significant increase in oxidative stress, while the g7-NP-Cur treated group expressed a strongly significant reduction. All values were normalized to the untreated control. For each condition 10 optical fields were measured, n = 10. (Bonferroni post hoc test revealed strongly significant differences between control and control with increased oxidative stress, and control and g7-NPs-Cur treatment group, one-way ANOVA: $F = 18.542$, $p < 0.001$). Permission for reuse kindly granted by Elsevier (Barbara et al., 2017).

We conclude that free Curcumin and Curcumin loaded NPs can significantly reduce oxidative stress *in vitro.* This reduction is to a lower level than the untreated control indicating that Curcumin has preventive effects as a radical scavenger even before severe oxidative stress is introduced. ROS could be attributed to cell damage in many pathologies including AD. The continuing reduction of free radicals is expected to reduce cell loss caused by chronic inflammation.

3.14. Summary of the results

The physicochemical properties of the NPs are well within the desired ranges for size, charge and PVA content. The Curcumin load is released within three days which demonstrates the biodegradability and the sustained release of the investigated NPs.

The safety of the NPs was shown using cell viability assays and biochemistry. There was no increase in necrotic or apoptotic cells after NP treatment *in vitro*. No PARP activation as sign of DNA damage could be detected. Curcumin showed a trend to be cytoprotective.

The anti-amyloidogenic properties of Curcumin could be shown with a Dot blot, a Western blot and a Thioflavin T based Aβ aggregation assay. NP-Cur can not only inhibit Aβ aggregation but also disrupts existing plaques *in vitro*. The mean number of plaques could be reduced with Curcumin treatment.

The results show how the g7-NP-Cur can rescue cells from Aβ induced inflammation. Cells treated with Curcumin expressed higher levels of IκB, which acts anti-inflammatory as the inhibitor of NFκB. Accordingly, to a reduction in inflammation less ROS could be detected, showing a decrease in oxidative stress. The anti-inflammatory and antioxidant properties could have promising effects in many chronic inflammatory diseases, including AD.

NP formulations with the g7 ligand were actively taken up into neurons via clathrin-dependent endocytosis indicating their ability to cross the BBB. The co-localization with plaques shows Curcumin's affinity for Aβ.

The investigated NPs are a safe transporter for targeted drug release. We expect the Curcumin to be an effective and well tolerable drug for AD prevention and treatment in all stages. The sustained release can be used to maintain an effective dosage over a prolonged time. The QD's ability to be tracked *in vivo* could help to design a theranostic NP that is both screening tool and therapeutic agent.

4. Discussion

The global burden of Alzheimer's disease has been estimated to affect 24 million people in 2013. This number could reach as much as 100 million by the year 2050 (Reitz and Mayeux 2014). AD is the most common cause of dementia. There is a clear trend towards a populational aging around the world. The improving treatment of cardiovascular and neoplastic diseases leads to a higher life expectancy especially in industrialized countries. In developing countries, the populational aging is rather caused by improving nourishment and hygienic conditions as well as access to medical and technological advancements. The global life expectancy increased from an average of 48 years around 1950 to 68 years in 2010. While more people live to reach old age, there is a decline in fertility rates from 5 children per women in 1950 to about only 2.2 in 2010. Additionally, there is the aging baby boom generation (Bloom et al. 2011).

The number of AD patients is expected to increase strongly since most of the patients affected are over 65 years old with more people reaching this age constantly. The number of unreported cases is expected to be quite meaningful since many people do not have access to medical treatment and hence do not receive the diagnosis of Alzheimer's disease. Furthermore, it is common belief that mental decline is a normal part of aging. This and the fact that there are neither sufficient diagnostic tools nor therapeutic measures leads to many potential patients not demanding medical treatment. It must be mentioned that any kind of neurological deficit must be examined by a neurologist since there is always the differential diagnoses of infectious, neoplastic or vascular diseases.

Even though it is pleasant to see many humans live long lives, it also demonstrates the urgent need to fight not only the physical weakening but also the neurodegeneration of old age.

Alzheimer's disease is a disease of the old with less than 5% of the patients being younger than 65 years (early onset AD). Three different genes APP, PSEN1 and PSEN2 have been linked to Aβ aggregation and early onset AD. Mutations in these genes can be inherited and lead to familial accumulation. On the other hand, they could be used as biomarkers.

It is believed that the AD pathology starts up to 20 years before the first symptoms appear. Since it is a chronic neurodegenerative disease accompanied by inflammation and cell death it is crucial to start treatment as early as possible. Today the only secured diagnosis can be obtained by visualization of plaques and tangles post-mortem. This is only of academic value and may not be used to screen for patients suitable for preventive treatment. The measurement of Aβ in the cerebrospinal fluid cannot be used as biomarker because it is often to be found in healthy individuals, as well. The research for diagnosis and treatment are therefore equally important.

Primum non nocere, which is Latin for "first, do no harm" is one of the most fundamental principles of bioethics. It originates in the Hippocratic Oath about 2500 years ago and emphasizes that no healthcare measure should harm the patient. If there is a threat to one's life, then potentially dangerous treatment can be endorsed when not acting would be even more harmful. An example would be the immediate surgery of a patient with a massive bleeding. In the case of AD there is no such need to act quickly and aggressively. Consequently, the treatment should not harm the patient in any way. Fortunately, we could not detect toxic effects of any investigated NPs *in vitro* using different viability assays and biochemistry. There was even a trend that Curcumin is neuroprotective by radical neutralization.

Other groups agree with our findings that neither the NPs with their different ligands, nor the Curcumin load are toxic. It has been shown that PLGA NPs are non-toxic both *in vitro* and *in vivo*. They do enrich in liver, brain, kidney and spleen but do not cause pathologies in these tissues (Semete et al. 2010). There is no threat of pathological accumulation if the half-life of the NPs applicated is considered. This varies depending on the exact composition of the NP and must be specified. It has been shown that the 50:50 ratio of PLA and PGA displays the fastest rate of biodegradation. An *in vivo* study showed this half-life to be around ten days with only a few traces still detectable after two months (Visscher et al., 1985). PEGylated and Rhodamine dyed PLGA NPs have also been shown to cause no toxicity in retinal pigment endothelium as well as human retinal vascular endothelium *in vitro*. This kind of drug delivery system did not just show the lowest toxicity but also the highest uptake into cells and organelles (Lin et al. 2017).

The safety of Curcumin has been demonstrated *in vitro*, *in vivo* and in phase 1 clinical trials in humans (Chainani-Wu 2003), (Chandran and Goel 2012).

There is epidemiological data that includes the lifelong consumption of Curcuma as a spice by a large group. The majority of Curcuma is being cultivated and consumed in south of Asia. While all Asian cultures know it as a spice and drug it is especially popular on the Indian subcontinent, where it is one of the main ingredients of the traditional Curry spice. Even though bioavailability and BBB crossing are known to be limited after oral intake it is still interesting to know if the lifelong and regular consumption of Curcuma has effects on one's health.

For more than 1 billion people especially on the Indian subcontinent as well as in the Middle East and some African countries Curcumin has been used as traditional medicine for thousands of years. There are different forms of application ranging from teas or other drinks, meals, pastes and other forms. While it is used in almost any field of disease it is most common in inflammatory and painful conditions like joint pain or skin ulcers. Since the antioxidation correlates with less DNA damage Curcumin intake has also been associated with a smaller risk of developing colorectal and prostate cancer, as well as a positive trend in other cancers. There is some good evidence that this polyphenol also has positive effects on the profile of blood lipids as well as glucose management, facing the big groups of cardiovascular and metabolic diseases (Kresno and Ernie 2008), (Wickenberg, Ingemansson, and Hlebowicz 2010).

The prevalence is the epidemiological number of people affected at a certain time point in a defined population. The world Alzheimer's report 2015 combined different studies to calculate the estimated prevalence of AD in western Europe of those between 65 – 69 years of age to be around 2.6% (Prince et al. 2015). Another group investigated the prevalence of AD of those older than 60 years in a rural area in India to be around 1.36% (Chandra et al. 1998).

In a second study it was investigated if there is a difference in the incidence of AD for those older than 65 years comparing the USA with the Indian population. The incidence describes how many people in a population are newly affected in a certain time frame, often one year. It has been proposed that the incidence rate per 1000 person-years is 3.24% in India while it is 4.7% in the US (Chandra et al. 2001), (Prince et al., 2015).

These epidemiological results should be interpreted carefully since correlation doesn't necessarily imply causation. This means that Curcumin could possible protect some Indian people from AD, but the differences could also be due to genetic, sociocultural or lifestyle differences.

As mentioned before, the issue with oral Curcumin intake is the low bioavailability and poor BBB crossing. To solve this problem the NPs used in this thesis were PEGylated for improved water solubility and had a g7 peptide coating to initiate BBB crossing. The team around Zhang had a similar approach and came to comparable conclusions. They had PEGylated PLGA instead of the PEGylated QDs used in our study. A small peptide called TGNYKALHPHNG (TGN) mediates the blood-brain barrier crossing in their study. Interestingly, the peptide QSHYRHISPAQV (QSH) has shown affinity towards Aβ and could possibly increase the NP concentration around plaques (Zhang et al. 2014). This search for ligands with high affinity for Aβ is important. With their help NPs can be designed to enrich near plaques, potentiating their positive effects while decreasing side effects. We tried one batch of NPs with an anti-Aβ antibody conjugated to the surface (data not included). While they did show affinity for plaques *in vitro* they also had some disadvantages. Antibodies are big and expensive proteins that strongly increase both the size and the price of the NPs. Small NPs are desired since it had been shown that NP toxicity increases with size. A preventive treatment should be relatively cheap because it becomes more effective when it is available to a large part of the population.

To prevent disease progression in early stages it is crucial to establish a screening tool for AD. With more than 95% the clear majority of AD patients do not receive a diagnosis before reaching the age of 65 years. This late onset AD is associated with mutations in the APOE gene alleles. Having a first degree relative with late onset AD will approximately double one’s risk of developing the disease (Reitz and Mayeux 2014). For most AD patients there is no biomarker and no certainty about inheritance making it challenging to differentiate between AD or other forms of dementia. There is not only the need for therapeutic innovations but also for diagnostic ones. The very long preclinical stage should be taken into consideration as the pathological deposition of both extracellular Aβ plaques and intracellular tau fibrils is expected to begin years to decades bevor the first symptoms appear. Therefore, the treatment should be well tolerated with limited risks and side effects because it must be administered over a long period of time.

PLGA as a well-known and non-toxic polymer has been used in surgical suture materials for decades and is not associated with strong adverse effects. Curcumin has been shown to be not only non-toxic but is also associated with positive effects like antioxidation which even results in a cytoprotective effect. The therapeutic agent should be able to relieve the existing pathologies and be preventive against new build up. We could show that Curcumin does not only inhibit the formation of higher order Aβ aggregates but also disassembles existing plaques. It has already been revealed that Curcumin can decrease the concentration of Aβ by suppressing the maturation of the APP (Zhang et al. 2010). Additionally, our results showed that the anti-inflammatory and antioxidant properties of Curcumin can rescue cells from cellular stress. To monitor these effects, the QD signal could be used for a feedback about the location of the NP enrichment.

Another big issue is the need for a diagnostic tool able to detect early stages of this chronic neurodegenerative disease. The pathology is assumed to develop over the time span of more than a decade making it highly desirable to detect and treat patients before they show severe symptoms. QDs on the NP's surface are highly specific and non-toxic fluorochromes that can be used for *in vivo* detection. We could show that QDs on NPs do not increase their toxicity. QD signals greatly co-localized with the neurons. It has been shown that QDs can even be tracked *in vivo* enabling the design of NPs that function as drug carriers and imaging agent, simultaneously (Gazouli et al. 2014).

When the QDs detected *in vivo* show a plaque like arrangement focusing on hippocampal and frontotemporal areas it could be a sign towards the development of Aβ plaques. In that case the NPs function as a screening device while also carrying the therapeutic Curcumin load. A positive plaque detection should be faced with the repeated application of Curcumin loaded NPs and control screenings for plaque development. A negative screening can be repeated after a longer interval or once symptoms appear.

The QDs used for our study were PEGylated, which is the conjugation of polyethylene glycol (PEG) to the QD's surface. The first advantage is that the complete NP becomes more water soluble since PEG is a hydrophilic molecule. Even more important is that PEG can mask the NP from opsonization and degradation by the immune system to some extent. Opsonization is the process in which a foreign material is flagged by antibodies and complement factors. Since the PEG is occupying much of the surface there is less space exposed for opsonization.

PEG being a neutral molecule also electrically isolates charged NPs. This is important, because charged surfaces are more likely to bind certain proteins and it has been shown that more neutral NPs are significantly less encountered by the immune system (Roser, Fischer, and Kissel 1998). It has been published that PEGylation of QDs can reduce their uptake by the reticuloendothelial system (RES) by the factor 6 and the uptake by liver and spleen by the factor 3 (Schipper et al. 2009).

QDs as well as PLGA NPs have the ability to bind random serum peptides on their surface. This should be topic of further research since the aggregation of peptides can be promoted or inhibited depending on the materials, surface charge and other variables of the NPs or QDs (Cabaleiro-Lago et al. 2010), (Vannoy and Leblanc 2010).

QDs can be more than tiny fluorochromes. Dihydrolipoic acid conjugated QDs have been shown to reduce the fibrillation of Aβ (Thakur et al. 2011). With only 2.5 nm in diameter they are about 100 times smaller than many commonly used NPs. This could have some promising effects since many processes like cellular uptake, active transport and toxicity are size dependent.

The Curcumin loaded and Quantum Dot coated Nanoparticles show the potential to be used as a theranostic tool making them suitable for both diagnostic and therapeutic applications. With the help of the g7 peptide the NPs are actively taken up into the CNS. A possible conjugation of the NPs with an anti-Aβ antibody could result in even greater localization at the plaques. This has been shown when the conjugation of iron oxide NPs with an anti-Aβ antibody resulted in a plaque like arrangement of the NPs. The NP immobilization next to the plaque increases the local concentration of the active component and can therefore reduce the necessary dosage (Skaat et al. 2013). Another study suggested Curcumin loaded magnetic NPs as a possible screening device for AD. Curcumin's affinity to Aβ resulted in a plaque like arrangement of the magnetic NPs that could be detected using the radiation free MRI scanner. This is desirable for a screening tool since no harmful radiation is emitted. The localization around the plaques could be confirmed by closer examining of the mouse brains after MRI scanning (Cheng et al. 2015). Similar NPs have been described to be detectable using either magnetic resonance imaging (MRI) or fluorescence microscopy (Veiseh et al. 2005).

Antibodies can also provide a trap effect, because once they have specifically bound to their targets they stop the NPs from further diffusion. This has been shown when the conjugation of PLGA NPs with an anti-transferrin and/or an anti-Aβ antibody resulted in significantly more crossing of the BBB and enrichment within the CNS (Loureiro et al. 2016).

Today the AD diagnosis is just as challenging as the treatment. The Aβ-PET (positron-emission tomography) can detect Aβ plaques *in vivo* after injection of a radioactive tracer (Dal-Bianco 2017). The issue is that both the tracer and the PET itself cause an increase in ionizing radiation. This is a non-desirable trait for a screening tool that is often used on healthy individuals and in early stages. Screening devices should be relatively harmless, so it is ethically acceptable to test large cohorts of possibly healthy people. Another approach regarding biomarkers has been proposed. Amyloid-beta derived diffusible ligands (ADDLs) in AD patients' cerebrospinal fluid are more concentrated then in healthy humans but still not enough to be detected by ELISA (Enzyme-linked Immunosorbent Assay). NPs conjugated with an anti-Aβ antibody are used to detect ADDLs more sensitively (Keating 2005).

The *in vitro* effects of Curcumin could partially be confirmed *in vivo*. Tg2576 mice were orally treated with Curcumin for 6 months starting at the age of 8 months. Immunocytochemistry of brain sections showed a significant reduction of the plaque burden while a Western blot illustrated a significant reduction of phosphorylated tau. Additionally, there was an increase in the anti-inflammatory cytokine Interleukin-4. A reduction of both soluble and insoluble Aβ could be observed as a trend (Shakeri and Sahebkar 2016). These findings are greatly suitable with our observations *in vitro*.

Similar results were made when Nanoliposomes with Curcumin on their surface were tested regarding their effects on Aβ. It is reported that Aβ secretion was reduced and overall Aβ toxicity was partially rescued. The group also showed that Curcumin does not only label Aβ plaques *in vitro* but also *in vivo* (Lazar et al. 2013).

Current literature states promising applications for loaded NPs for both therapeutic and diagnostic purposes. For AD a theranostic approach is desirable since it will enable the treatment and monitoring of the disease with only one application. For the highly aggressive brain tumor glioblastoma multiforme it has been shown that Theranostic Photonic Nanoparticles (TPNs) loaded with Curcumin are able to cross the BBB *in vivo.*

The TPNs could be shown to enrich in the tumor of a mouse model and could therefore be used to monitor the tumor progression. The same TPNs had been shown to reduce the overall number of malignant glioma cells *in vitro* (Singh et al. 2016).

A way to increase the local concentration of the NPs at the required site is to conjugate tissue specific antibodies to their surface. Gastric cancer mouse models were injected by fluorescent antibody conjugated NPs. After tail vein injection the NPs greatly co-localized with the tumor as detected with an MRI scan. In this case the antibody was both the targeting ligand and the active drug. A gastric cancer specific ATP synthase subunit antibody was used that resulted in the enrichment of NPs at the tumor as well as an inhibition of growth by blocking the mentioned ATP synthase (Wang et al. 2014). This approach could be promising in AD treatment since Aβ targeting and the inhibition of further aggregation are highly desired goals. For this thesis we investigated anti-Aβ antibody conjugated NPs for their plaque affinity. The data is not included as it seems that the moderate concentration of 40 pmol of anti-Aβ antibody in each 15 mg batch of NPs, did not overcome the Aβ affinity of the Curcumin. This should be subject of further investigation. A possible downside could be the large size of antibodies that might either increase the size of the NP or decrease the concentration of the loaded drug. On the other hand, the highly specific targeting could intensify the desired effects while minimizing the side effects. Many different pathologies are limited to very specific tissue types and hence only affect a small part of the whole body. Conventional oral pharmaceuticals often distribute across a complete compartment or engage specific chemical properties for example water rich environments when being hydrophilic. This usually means that only a very low percentage of the administered drug reaches its goal. To reach an effective concentration, a large dosage is needed that inflicts undesired effects. With a targeted therapy a bigger fraction of the compound reaches the required site of action, enabling lower doses and less adverse effects.

In a different study NPs were loaded with the neuroprotective agent 3-n-Butylphthalide and conjugated with Fas ligand antibodies. Fas receptor activation induces apoptosis in many cases of injury including ischemia. The group could show *in vivo* that the drug loaded NPs could rescue more cells from ischemia induced cell death then the administration of the drug by itself (Lu et al. 2014).

NPs can also deliver Curcumin to the required site and prolong its effects by sustained release. The activity of free Curcumin has been shown *in vivo.* Curcumin did not only label the Aβ plaque, proving its affinity but also disrupted them. Additionally, the group observed distorted neurites to restore partially (Garcia-Alloza et al. 2007). In tune with our findings, other groups showed Curcumin not only inhibiting the formation of new Aβ aggregates but also disaggregating existing ones (Yang et al. 2005). It has also been shown to label senile plaques *in vitro* and *vivo* (Garcia-Alloza et al. 2007).

One obstacle of NP treatment is the parenteral administration that usually requires professional assistance. Creating long lasting NPs that target specifically is a possible way to lower the frequency of administration. Options that do not require the i.v. or i.p. injection of NPs are being tested. It has been shown that NPs can reach the brain after non-invasive pulmonary administration (Sancini et al. 2016). This could enable the application via inhaler, which is non-invasive and hence well suited for long-term use. Inhalers have been used in the therapy of asthma for decades.

Aβ fibrillation is trace metal dependent and can only form aggregates in the presence of zinc, copper and iron. Transgenic mice without synaptic zinc do not deposit Aβ. Metal chelation *in vitro* stopped Aβ aggregation (Huang et al. 2004). We did not investigate the metal chelation properties of Curcumin in this study, but two other groups published articles regarding this topic. It was found that Curcumin significantly binds to Fe^{2+}. It was also discovered that Curcumin bound to iron is less likely to be taken up by cells (Messner et al. 2017). This is especially promising regarding AD, because Curcumin could bind iron and then remain extracellular to perform its antioxidant and anti-amyloidogenic properties at the site of the plaque. This is supported by the findings that Curcumin is a metal chelator for Mn^{2+} and Zn^{2+} and still has antioxidant properties when bound to metal ions (Mary, Vijayakumar, and Shankar 2018). Additionally, a zinc-dependency of Aβ induced synapse loss has been shown (Grabrucker, Schmeisser, et al., 2011).

Tau aggregation has not been investigated for this study, but other groups suggested that Curcumin can suppress tau accumulation and even induce its clearance by molecular chaperones. This even lead to behavioral improvements of tau transgenic mice (Ma et al. 2013). These publications indicate that Curcumin could be a potential drug to treat both the Aβ and the tau pathology of AD.

Curcumin and NPs are suitable for the treatment of many other diseases as well. This can be either a positive side effect in the AD treatment of multimorbid elderly patients or a completely different approach.

It has been investigated if poloxamer 188-coated PLGA NPs can transport the chemotherapeutic doxorubicin into human glioblastoma cells. In tune with our results, clathrin-dependent endocytosis was observed (Malinovskaya et al. 2017). Glioblastomas are highly aggressive WHO grade 4 tumors that even if treated only allow a mean survival of around 15 months. The surgical removal of the tumor is not very effective because tumor cells spread early in the disease. Specific NPs can be used to target these micro-metastases and could possibly potentiate the effects of radiation therapy.

NPs cannot only deliver medication but also heat. In a recent study iron-oxide NPs were injected directly into the adenocarcinoma of a mouse model. An AC magnet was applied on the skin close to the tumor. A thermometer probe inside the tumor measured temperatures over 50°C which had a cytotoxic effect on tumor cells without having relevant systemic side effects (Pearce, Petryk, and Hoopes 2017). Similar approaches using laser have been published, as well (Espinosa et al. 2016).

Thermal applications through NPs are not limited to cancer treatment but are also tested for the disintegration of undesirable deposits. Gold NPs heated up using weak microwave radiation have been shown to dissemble Aβ aggregates into smaller oligomers (Bastus et al. 2007).

Behavioral improvement regarding spatial memory could be measured in rats receiving Tacrine loaded Chitosan NPs. Tacrine is a cholinesterase inhibitor and is FDA approved for the treatment of AD. Even though NPs were injected peripherally, the hippocampal APP expression was reduced significantly, indicating sufficient BBB crossing (Hassanzadeh et al. 2016).

Curcumin has been recommended for a variety of disorders with the only known side effects of mild intestinal problems like nausea or diarrhea. There has been a clinical trial with patients receiving up to 8000 mg/day over a time of 3 months with no toxic effects to be found (Ann C 2000). People susceptible of bleeding, low blood-pressure and kidney stones should reconsider the Curcumin treatment since it could aggravate these disorders.

For most other patients Curcumin can be recommended as a preventive dietary supplement even before the first AD symptoms appear. The absence of severe adverse effects enables the treatment of chronic diseases without the fear of inducing other undesirable effects.

Many other publications are consistent with our findings that Curcumin as well as Curcumin loaded Nanoparticles down regulate the expression of NF-κB. Since NF-κB is one of the central transcription factors of inflammation, its down regulation has shown therapeutic effects in different diseases. In the case of AD, we could show that NP-Cur could significantly reduce the degradation of the inhibitor of κB. This resulted in less inflammation leading to a lower secretion of ROS. Overall, we observed that NP-Cur can partially rescue cell loss caused by the Aβ peptide.

The oxidative stress caused by ROS damages cells in all stages of AD and is therefore a great point of encounter. Especially mitochondrial DNA is being oxidized in AD which had been quantified to be 10-fold higher than the oxidation of nuclear DNA (Wang et al. 2005). Curcumin's ability as radical scavenger may not only delay neurodegeneration but many other sites of oxidative damage. It has been shown that cardiac reperfusion damage is caused by oxidative stress as well as mitochondrial dysfunction and it can partially be rescued with a pretreatment with Curcumin (González-Salazar et al. 2011). AD, cancer and cardiac ischemia are all rather diseases of the old age. The safe and easy preventive application of Curcumin is expected to have positive effects on these and many other frequent diseases. This still must be tested thoroughly, since it is not excluded if there are strong contraindications.

The c-Jun N-terminal kinases (JNK) is a signaling pathway involved in inflammation and apoptosis. The JNK activation has been linked to the degeneration of neurons in AD (Zhu et al. 2001). Curcumin has been shown to inhibit the JNK activation after different means of cellular damage. Like in this study the Curcumin concentration was found to be effective on a scale of around 10 μM (Chen and Tan 1998).

Curcumin's low resorption after oral intake is promising regarding inflammatory bowel disease since the medication partially remains within the intestine. It's been reported that patients with Crohn's disease and Ulcerative Colitis had clinically improved and expressed less serological markers of inflammation after being treated with 550 mg of oral Curcumin twice a day for two months (Holt, Katz, and Kirshoff 2005).

The inhibition of TNFα by Curcumin loaded lipid nanocarriers could be confirmed in mice with colonic inflammation (Beloqui et al. 2016).

Another widespread disease going along with chronic inflammation is the rheumatoid arthritis. Patients report severe joint pain during movement which often leads to reduced activity with a higher risk of obesity and the related secondary diseases (Albrecht et al. 2016). Chronic inflammation can intensify psychological stress and has been linked to higher rates of depression and anxiety among patients with rheumatoid arthritis (Matcham et al. 2016). There are medical options for treatment but they often involve distinct side effects. A clinical study could show that the intake of 500-1000 mg of Curcumin daily for 90 days could not only reduce clinical symptoms but also the levels of C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) which are both serological markers of inflammation (Amalraj et al. 2017).

Inflammatory pathways do not only play a role in chronic inflammatory diseases but also in many cancerous tissues. NF-κB is expressed in most human tumors suppressing apoptosis in many of them. It's been discovered that Curcumin can suppress many NF-κB related gene products, including cyclooxygenase-2, prostaglandin E_2 and interleukin-8 which all previously have been linked to pancreatic tumor growth (Li et al. 2004). A clinical phase II study showed cases where tumor lesion size could be decreased with Curcumin treatment (Dhillon et al. 2008).

Nude mice with human breast cancer often develop NF-κB dependent lung metastases. It has been shown that Curcumin or Curcumin + Taxol can significantly reduce the percentage of mice with lung metastasis while also increasing cell viability (Aggarwal et al. 2005). On the other hand, it should be mentioned that some chemotherapeutic agents act by generating ROS. We could show that Curcumin significantly reduces ROS *in vitro*.

Another group observed that Curcumin reduces cyclophosphamide-induced tumor regression by alleviating ROS stress (Somasundaram et al. 2002). We conclude that the indications of Curcumin just like any other drug must be evaluated before administration.

In the treatment of prostate cancer cells, it has been published that Curcumin can potentiate the effects of immunotherapy mediated by TRAIL (tumor necrosis factor related apoptosis-inducing ligand) (Deeb et al. 2003).

In lung carcinogenesis Curcumin has been shown to have synergistic effects with Resveratrol, a polyphenol found in red wine. The result was the activation of tumor suppressor gene p53 by hyper-phosphorylation (Malhotra, Nair, and Dhawan 2014).

The literature states many different possible Curcumin applications like in Diabetes, Psoriasis, Lupus, Biliary Dyskinesia, Recurrent Respiratory Tract Infections and many more (Gupta, Patchva, and Aggarwal 2013). The positive effects on many populational diseases and the negligible side effects of mild intestinal irritation makes Curcumin a possible candidate for widespread administration.

Finally, there has been one study greatly agreeing with our findings. It could be shown that Curcumin labels Aβ plaques *in vitro* on incubated brain sections. *In vivo* it was observed that mice peripherally injected or fed with Curcumin showed less Aβ aggregation. Existing plaques were disrupted, and the formation of new plaques was inhibited (Yang et al. 2005). This is especially promising because the low concentrations of as little as 0.1 μM after oral administration already had positive effects. This indicates a potentiated effect when higher concentrations are maintained using targeted Curcumin loaded NPs.

We conclude that the investigated NPs are safe and do not cause severe toxicity. Their sustained release and specific targeting allows the formulation of NPs that maintain an effective dosage at the desired site. Curcumin can alleviate the inflammation and oxidative stress caused by Aβ aggregation. Moreover, it is able to reduce the Aβ aggregation itself, by inhibiting plaque formation and disruption of existing plaques. The neuronal uptake indicates affinity towards neurons and ability to cross the BBB mediated by g7. The QDs ability to be tracked *in vivo* could be used in a theranostic approach in which the NPs transport the Curcumin into the CNS while the QDs are used to monitor the disease progression.

The BBB crossing has been shown before when different compounds were both stabilized and transported into the brains of rodents with the help of NPs (Grabrucker et al., 2016) (Valenza et al., 2015).

A *in vivo* study using an AD mouse model should be followed to confirm and complement these *in vitro* findings.

Conclusion

Most AD patients do not develop symptoms before the age of 65 years and populational aging results in more people reaching this age globally. This pleasant trend has some effects that should be monitored consequently. It has been estimated that by the year 2050 as many as 2 billion people could be 65 years and older, making up between one fourth and one fifth of the total human population (Bloom et al. 2011). This development shows the urgent need to find a causal therapy to combat neurodegeneration and AD especially as the most common cause of dementia. Equally important is the establishment of a safe, specific and sensitive diagnostic tool. This should not only be used to secure the diagnosis in symptomatic patients but also to screen healthy individuals since it is known that the pathologies of AD start decades before the clinical stage. A screening tool however must be safe and relatively cheap, so it can be used on large cohorts. The same accounts for the therapeutic agent as well, because the application over many years should not induce other health issues or severe side effects.

We believe the theranostic approach with Curcumin loaded, Quantum dot modified and targeted Nanoparticles to be very promising. We were able to show their safety *in vitro* with a trend towards being cytoprotective with the antioxidant effects of the Curcumin. The NPs were able to induce clathrin-dependent endocytosis enabling BBB crossing and enrichment within the CNS. It had been shown before that the g7 ligand can increase the concentration of the NPs inside the brain of rodents.

The PLGA matrix of the NP is known to be non-toxic. This biodegradable polymer can be greatly modified to alter its affinity and half-life. For the 7-day treatment the Curcumin loaded Nanoparticles were superior to free Curcumin, indicating the advantage of sustained release.

The QDs can be tracked *in vivo* without the use of ionizing radiation and are thereby suitable as a non-toxic screening tool. Theranostic NPs modified with QDs could be used to monitor disease progression while also delivering medication to the site of pathology.

The Curcumin did not only show affinity for Aβ by co-localizing with the plaques, but also showed important properties regarding the pathology. We showed the Curcumin load to be anti-amyloidogenic, anti-inflammatory and antioxidant. Curcumin inhibited Aβ aggregation and disrupted existing plaques, as well. The total number of plaques was reduced by the treatment. There was a trend towards a rescue on cellular level and a higher synapse count after only 7 days of treatment in a disease that usually extends over years.

The Aβ aggregation in AD causes neuroinflammation which then leads to increased oxidative stress that damages cells. Curcumin encounters all three steps of this pathology, potentiating its effects.

We conclude that NP formulations could be used to diagnose, treat and monitor the pathological Aβ aggregation in AD patients. *In vivo* studies with AD mouse models are required to confirm our findings. NP targeting could possibly be improved with the conjugation of specific antibodies and has possible applications in many other diseases.

It is especially pleasant to mention that Curcumin does not only have very little side effects but can also be used to treat many other populational diseases. Many elderly people are suffering from multiple morbidities of which many involve inflammatory processes. With Curcumin many populational diseases could possibly be targeted with one safe and available compound.

We are pleased to state the safety as well as the anti-amyloidogenic, anti-inflammatory and antioxidant properties of the investigated NP-Cur and their affinity towards neurons *in vitro*.

5. Summary

In summary it can be reasoned that the aims of the thesis were met to a great extent.

The physicochemical properties of the Nanoparticles (NPs) show size, charge and polyvinyl alcohol content that are well within the desired ranges. The complete Curcumin release within three days indicates the biodegradability of the investigated NPs. The sustained release out of the NP load can be used to maintain an effective dosage over a longer period.

Our main concern was the safety of the NPs, their different ligands and the Curcumin load. Cell viability assays showed that neither the free Curcumin nor the NPs resulted in an increase of apoptosis or necrosis over 1 or 7 days. These findings are supported by biochemical investigations. The cell lysates of cells treated with our different NP formulations for 1 or 7 days showed no differences in the ratio of the poly (adenosin diphosphate-ribose) polymerase (PARP) and cleaved PARP when compared to the control. We could not detect toxic effects *in vitro* and there were trends towards the cytoprotective effects of Curcumin.

We investigated Curcumin's beneficial effects after being released from the PLGA poly(lactide-co-glycolic-acid) NPs. It was observed *in vitro* that the g7-NP-Cur significantly increased the levels of the inhibitor of kappa B (IκB) which is known to have an anti-inflammatory effect. Curcumin significantly rescued the cells of the inflammatory response mediated by the preincubation with Amyloid beta (Aβ). Empty NPs had no effect on IκB. The CellRox® detection kit was used to measure reactive oxygen species (ROS). Curcumin significantly rescued cells from the oxidative stress introduced by the Fenton's reaction. It could even minimize the ROS level to a smaller value than in the untreated control that did not endure the preincubation with the Fenton's reagents. The same was observed for Curcumin loaded NPs over 7 days. Decreasing oxidative stress is of great interest in Alzheimer's disease (AD) treatment because ROS are known to play a major role in the mechanisms of cell damage. Using an original conformation (OC) Aβ antibody for a Dot Blot experiment it was shown that Curcumin and NP-Cur could significantly inhibit the formation of OC Aβ aggregates. A Western blot for higher order Aβ aggregates confirmed these results.

A Thioflavin T β - Amyloid (1 - 42) Aggregation Kit revealed that g7-NP-Cur can not only inhibit Aβ aggregation but also disrupts existing plaques. The mean number of plaques could be reduced by the treatment, as well.

Additionally, the NPs were actively taken up into neurons via clathrin-dependent endocytosis indicating their ability to cross the blood-brain barrier (BBB). The NP-Cur co-localized with the Aβ plaques showing Curcumin's affinity for this pathologically aggregating peptide. Targeted release results in a higher fraction of the NPs reaching the site of action. This way the effects are potentiated while the side effects are minimized.

Therefore, we conclude that the investigated NPs are a safe transporter for targeted drug release. We expect the Curcumin to be an effective and well tolerable drug for AD prevention and treatment in all stages. G7 ligands help to initiate BBB crossing. Furthermore, the Quantum Dot's (QDs) ability to be tracked *in vivo* could help to design a theranostic NP that is both screening tool and drug transporter.

Future applications for NPs are countless. NPs as drug carriers offer many advantages over the application of the drug alone. The NPs can protect their load from opsonization and degradation. NPs can carry compounds that are not suitable for oral administration. Targeted NPs can be designed to enrich within a desired tissue to maximize their effectiveness while minimizing peripheral side effects. The sustained release of the drug results in prolonged effects after a single administration. Magnetic NPs are tested for thermal ablation of tumors. Theranostic NPs can carry a drug while monitoring disease progression *in vivo*. The many possible NP formulations must be tested for toxicity and metabolism, as well as short and long-term effects.

Curcumin has been used for several desirable properties, like its anti-inflammatory, antibiotic and anti-cancerogenic effects. The antioxidative and anti-inflammatory properties are promising in many populational diseases that involve inflammation and oxidative stress, including AD. The anti-amyloidogenic qualities shown in this thesis could be used to alleviate the Amyloid beta burden in Alzheimer's disease.

Summarizing it can be argued that the tested NPs have a range of desirable effects *in vitro*. They are non-toxic drug carriers suitable for blood-brain barrier crossing. The Curcumin load shows antioxidative, anti-inflammatory and plaque disrupting properties.

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Curriculum Vitae

Personal information

Name:	**Johannes Robin Keller**
Year of birth:	1992
Place of birth:	Ulm, Germany
Citizenship:	German

Education

Tertiary

Clinical Medical School
Ulm University
2014 – 2019 Ulm, Germany

Preclinical Medical School
Regensburg University
2012 – 2014 Regensburg, Germany

Secondary

German University Entrance Diploma
Anna-Essinger Gymnasium
2003 – 2012 Ulm, Germany

USA High-School Diploma
Frankfort-Schuyler Central High-School
2009/2010 Frankfort NY, USA

Primary

Meinloh Elementary School
1999 – 2003 Ulm, Germany

Professional Experience

Medical Education Research Project Assistant, Ulm University, 06-10.2017

Intensive Care Unit internship, Charité Berlin, 03/04.2017

Nanomedicine lab training, Modena University, Italy, Giovanni Tosi, 03/04.2016

Radiologist Assistant, Radiology Parkstrasse Ulm, 09.2014-01.2015

Neurological EEG Assistant, Hospital Barmherzige Brüder Regensburg 06.2013-06.2014

Professional Memberships

German Scholarship Foundation

Neuro Track, Ulm University

Experimental Medicine Track, Ulm University

International Graduate School for Molecular Medicine, Ulm University

Teaching

Anatomical Tutor, Regensburg University, 2013/2014

Biochemical Tutor, Ulm University, 2016/2017

Physics Lecturer, IFS student courses Nürnberg, 2016/2017

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Languages

Fluent **German**

Proficient **English**

Advanced **Latin**

Ulm, 2019 Johannes Robin Keller