Functional fluorescent materials and migration dynamics of neural progenitor cells

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INTRODUCTION

1.1. GENERAL BACKGROUND

Cells are the building blocks of life. Research at the single cell level is crucial as it allows to unravel the complexity of this smallest living unit. On the inside, one cell holds a whole world of molecular machinery within its membrane. On the outside, it interacts and communicates with its environment and other cells. For complex mammalian organisms, understanding and influencing cells on individual level is fundamental to understanding and influencing them at the higher level of tissue, organs, and as a whole.

The development of light microscopy was crucial as it enabled to uncover the structure and dynamics of cells. However, cells are mainly composed of water and are thus translucent. Only the cellular membrane, the heterochromatin inside the cell nucleus, as well as several organelles are dense enough to be visible by light microscopy. For more detailed analysis, molecules and structures can be tagged with fluorescent materials allowing them to be better detectable. Beyond the mechanism of simple fluorescent tagging, functional materials have been created with capabilities to sense the environment or influence cells. Popular are systems enabling e.g., calcium concentration indication

or measurements of voltage change across the cellular membrane [1]. These tools also drive fundamental research and biomedical innovations.

Numerous fluorescent microscopy techniques exist allowing information acquisition for different research requirements. For example, a light sheet microscope is useful to study tissue assemblies [2], whereby Stochastic Optical Reconstruction Microscopy (STORM) is useful for high-resolution of small structures like microtubules [3]. For research on the single-cell level, light fluorescence microscopy has been widely employed. Hereby the entire sample is illuminated, and the resulting fluorescence collected, thus gathering also light from the out-of-focus planes (i.e., below and above the focal plane). Particularly when imaging cells the resulting images are blurry, as cells contain a high volume of liquid that scatters light. Laser scanning confocal microscopy (LSCM) employs a pinhole that blocks the out-of-focus light and creates greater resolution and lower noise levels. However, as the pinhole only illuminates a small area, the whole sample needs to be scanned line by line. This process is time-consuming and can cause photo-damage to live samples.

The design of the spinning disc confocal microscopy (SDCM) improves these conditions. Instead of a single pinhole as in the LSCM, the SDCM system uses a spinning disc with thousands of pinholes in it. These are arranged in a way that every point in the field of view is covered when the disc rotates, resulting in a simultaneous and thus fast illumination of the whole sample. The pinholes are placed with interspace between them to avoid crosstalk between the “mini-beams” of light going through the pinholes. However, these interspaces also block most of the excitation light. In the Yogokawa disc this condition is improved by an additional disc containing an array of micro-lenses that match the array of the pinholes sample [4]. These micro-lenses collect and focus the excitation light thus improving the illumination of the. To collect the image information, charge-coupled device (CCD) cameras are used. These are two-dimensional detectors that simultaneously record the spatial as well as the intensity information of all mini-beams thus leading to high speed of image acquisition. This high temporal resolution allows imaging of fast dynamic processes. Combined with the reduced photodamage resulting from the pinhole design, the SDCM system is particularly advantageous for the imaging of dynamic processes and motility of living cells and is the main method that was employed in this thesis.

Despite the lower photodamage in the SDCM system, light exposure can still be damaging for cells. Next to the damaging effect of high intensity light, the main phototoxic effect often results from fluorophore photobleaching [5]. With each illumination of the fluorescent sample a fraction of the fluorescent probe is irreversibly destroyed and additionally generates free radicals and other reactive breakdown products. The best way to reduce photodamage is to reduce exposure time and intensity during excitation, while still retaining a useful signal-to-noise ratio. Additionally, good cell permeability is crucial to accumulate enough fluorescent material inside the cell and beyond that the probe should not interfere with the biological system. Thus, fluorescent probes need to fulfil a range of requirements to ensure a healthy cellular state.

A myriad of fluorescent probes has been developed, each trying to perform better than previous probes. Their performance is defined by various features: (1) Photostability - the capability to withstand photobleaching. (2) Signal intensity - the brightness of

the emitted light, specified by the extinction coefficient (fraction of absorbed light at a given wavelength) and the quantum yield (ratio of emitted photons relative to absorbed photons). (3) Biocompatibility – defined as the inertness of the material to cellular environment, without perturbations of its system. (4) Cellular permeability or uptake - the internalization of probes over cellular membrane. (5) Specificity or functionality – defined as how well the aimed target or intended function is achieved. Material development aims at fulfilling the listed requirements, whereby different probes are needed for the diverse research questions and applications. The current range of available fluorescent materials is enormous and includes organic molecules, synthetic dyes, quantum dots, metal nanoparticles, silica nanoparticles, organic and inorganic polymers, nanotubes, and nucleic acid-based materials [6].

Despite technological advances in the design of fluorescent probes, many still face challenges of strong background fluorescence, photobleaching, or limited cell permeability. Fluorophores at higher energy with short wavelengths of the visible spectra, i.e., ultraviolet (e.g., Hoechst 33342 with excitation at 405 nm) and blue (e.g., GFP with excitation at 488 nm), usually yield bright signals but also result in higher phototoxicity [7, 8]. These probes are great for short-term experiments or snap shots. Fluorescent probes in lower energy with higher wavelengths, i.e., green (e.g., RFP with excitation at 561 nm), red (e.g., Cy5 with excitation at 647 nm), often yield less bright species but also have a lower photodamaging effect [7, 9]. These probes are useful for long-term imaging and frequent time-lapse experiments. The "perfect" fluorescent probe for a given experiment is rarely perfect, but most often a balance between the requirements and current possibilities. Each of the available materials were created to fulfill certain functions and the development of better and more functional materials is ongoing.

The work presented in this thesis encompasses different topics, centring around confocal microscopy of fluorescent objects and living cells. In the first part of this thesis, two functional fluorescent materials are employed. In chapter 2 DNA-encapsulated silver nanoclusters (Ag-DNA) are used as an intracellular sensor. In chapter 3 polymersomes are employed as a long-term imaging probe in living cancer cells. Both materials are very interesting for a wide range of applications and an overview of the multifaceted research as well as background information is given in the following sections (1.2 and 1.3).

The second part of this thesis focuses on neural progenitor cells (NPCs). In chapter 4 various fluorescent probes are tested for the purpose of long-term visualization of the NPCs and their nucleus during imaging. Chapter 5 observes and analyses the migratory dynamics of NPCs on uniform and patterned structures utilizing fluorescent reporters. Spinning-disc confocal microscopy and time-lapse imaging allow to resolve the dynamics of cell machinery and are employed here to gather data on NPC dynamics. Furthermore, *in vivo*, NPCs are surrounded by nano and micro-sized structures providing attachment sites and influencing their behaviour. Thus, various patterning techniques have been developed to mimic these structures *in vitro*, two of which are employed in this thesis. NPCs are in the focus of attention due to their inherent capabilities to regenerate the nervous system. Learning more about cellular behaviour and dynamics will provide more insights on organism development, as well as for treatment of central nervous system injuries and diseases. Background information on these fascinating cells is provided in section 1.4.

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1.2. DNA-TEMPLATED SILVER NANOCLUSTERS (AG-DNA)

1.2.1. AG-DNA BACKGROUND AND PROPERTIES

Metals are intriguing materials as their size and shape determine their electrical, magnetic, and optical properties [10, 11]. Due to nearly freely moving electrons in the overlapping outer shells of metal atoms, they are exceptional electrical conductors and optical reflectors. Oscillations in the electron charge density on the surface of the metal, known as surface plasmons, can be induced through photon or electron excitation. Reducing the size of the metal to nanoparticle dimensions (i.e., 1 to 100 of nanometres), enables interesting coupling interactions with fields, dependent on the dimensions of the particle, including fluorescence emission. When the metal size is further decreased and approaches the Fermi wavelength of electrons (about 0.5 nm for Ag and Au), the band structure breaks up into discrete energy levels [12]. Thus, these metallic nanoclusters are expected to resemble the energy levels of molecules, equipping them with molecule-like properties [13]. Consequently, these metals show strong photoluminescence and photostability [14] and provide the link between single metal atoms and plasmonic metal nanoparticles.

Nanoclusters comprised of silver have been in focus due to their brighter fluorescence than gold particles [12] and their intrinsic anti-bacterial properties [15]. However, various factors affect the stability of silver nanoclusters in solution, leading to agglomeration and oxidation [16]. Polyanionic molecules with high affinity for silver ions have been utilized to protect the clusters, yielding water-soluble and stable silver nanoclusters under physiological conditions [17]. Among them are (synthetic) polymers, phosphines, proteins, solid matrices, thiolates, and nucleic acid molecules; all leading to nanoclusters with different fluorescence spectra and varying quantum yields [13, 16].

The combination of silver nanoclusters and DNA molecule in particular yields an intriguing blend, producing a new class of nanophotonics [18]. The metal clusters have been indicated to be rod-shaped [19], with varying resonances yielding emission wavelengths from the blue to the near infrared spectrum [18, 20]. Remarkably, because the DNA stabilization determines the size and shape of the cluster, the fluorescence of this hybrid molecule can be tuned by the selection of the DNA length and sequence [21, 22]. The connection with DNA bases appears to have a significant effect on the intrinsic optical properties of the nanoclusters, as only specific configurations exhibit significant fluorescence.

Ag-DNA exhibit a strong variation in optical properties with characteristics including bright fluorescence, high photostability [23], and quantum yields over 90% [19]. The optical properties of Ag-DNA are not only highly dependent on the nucleobase sequence, but also on pH and temperature and ultimately their cluster size and shapes. Further, because the DNA configuration influences which species exhibit fluorescence, after synthesis, their fluorescence can still be tuned or switched on and off through the interaction with nearby DNA templates [24, 25]. All these characteristics make these nanomaterials valid competitors to standard fluorophores. Compared to organic dyes, Ag-DNA can be brighter and more photostable. Compared to quantum dots, they are smaller and can be less toxic and less prone to blinking on longer timescales [26].

Beyond that, nucleic acid nanotechnology has exhibited tremendous advances in its field, by using the molecules as architectural elements to build nanostructures with var-

ious functionalities [27]. For more targeted applications, any recognition sequence can be integrated naturally into the DNA template without the need of chemical modifications. Additionally, being a natural molecule, DNA is fully biocompatible. This increases its value as a stabilizing ligand and facilitates broad application.

DNA-stabilized silver nanoclusters (Ag-DNA) were first introduced by the Dickson group in 2004 (Petty 2004). Since then, these nanomaterials have been employed for numerous applications as fluorescent trackers. Particularly its sensitivity to environmental cues, expressed through shifts in the fluorescence spectra, makes Ag-DNA a valuable tool for sensing applications. It has been applied for the detection of genetic mutations [25, 28, 29], as well as low concentrations of metal ions [30, 31] and DNA/RNA molecules [24, 32]. Additionally, the tuneable optical properties and easy positioning make these structures interesting for applications in nanophotonics and optoelectronics [18, 33].

Beyond that, existing research is strongly focused on uncovering the structure of the Ag-DNA and understanding how the tunability of the fluorescence spectra arises. Despite this focus, these nanomaterials are poorly understood in their formation pathways and photo-physical properties as it is challenging to obtain structural information. The conformational states of Ag-DNA are usually short-lived, impeding measurements to uncover its secondary structure. RNA can also be implemented to stabilize silver clusters [34], but DNA is the more favourable choice due to its higher stability.

The synthesis of Ag-DNA is relatively fast (1h), low in cost, and easy. However, due to oxidation by air and photobleaching, as well as continuing chemical interactions, many species are short-lived (i.e., hours) [35], and only specific purified samples show high stability (e.g., a certain 28mer is stable for 30 days at room temperature) [36].

To synthesize Ag-DNA, silver salts (such as $AgNO_3$) are mixed in a specific ratio with DNA templates and subsequently reduced, typically with sodium borohydride ($NaBH_4$). Although cations generally bind to the negatively charged phosphate backbone of the DNA, silver ions (Ag^+) associate with the DNA bases [37]. Hereby, silver ions show a strong affinity order for the bases, preferring cytosine over guanine, followed by adenine, and at last thymine [36, 38]. Reduction with (NaBH4) leads to nucleation of the neutral silver core (Ag_0), whereby silver ions in the periphery remain, acting as “glue” and connecting the silver core to the DNA bases (Figure 1.1).

Notably, upon synthesis, an individual DNA template produces a heterogeneous mixture of distinct Ag-DNA conformations and silver cluster products. Many of these species, present in a single solution, are non-fluorescent, whereby only about 25% might show high fluorescence (i.e., signal considerably higher than noise level) [39].

Recently, methods have been developed to isolate individual species. Using high-performance liquid chromatography with in-line mass spectrometry (HPLC-MS), it is possible to select for pure Ag-DNA complexes and to identify their total number of silver atoms and silver cations [39]. This method yields spectrally pure and more stable fluorescent products and facilitates the continued research and application of Ag-DNA.

1.2.2. TEMPLATE DESIGN AND STRUCTURE

To increase the pool of fluorescent species, cytosine- and guanine-rich sequences were found to be important [40, 41]. However, using solely homopolymers of cytosine

or guanine produces species with low temporal stability [42]. Thus, a mixture is necessary to produce fluorescent products with longer stability. Long oligomers were found to yield products with higher stability and tend to generate bigger clusters [19, 39, 43, 44].

Beyond that, the understanding of how the composition of a mixed-base sequence determines the cluster formation, is still poor. The range of possible permutations is enormous. Considering using a short template with only 10 bases, already allows for over 10 Million possible sequence arrangements [42]. Thus, the design of DNA templates is still tedious as finding effective sequences is generally done by experimentally testing them.

With advanced understanding of the interactions between silver ions and DNA bases, machine learning can be used to predict sequences that stabilize fluorescent clusters. Copp *et al.* (2014) used pattern recognition to find "base motifs" which favour radiative over non-radiative decay. A "base motif" consists of 3-5 bases which are necessary to encapsulate a cluster. Examples for found motifs include GCG, CGC, and GG_AC for bright clusters, and TTG, ATT, and AT_T for dark clusters, confirming the necessity of guanine and cytosine bases for a fluorescent product. Employing this model and a learning algorithm, the authors were able to predict templates that produce bright Ag-DNA with 80% accuracy [42].

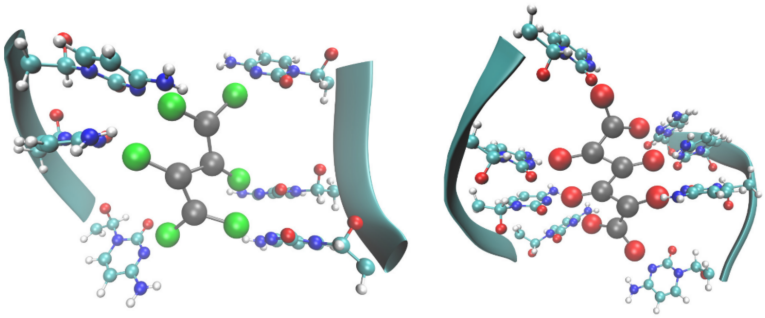

Figure 1.1: Illustration of Ag-DNA structures. Representation of Ag-DNA constructs containing four silver atoms (left) and six silver atoms (right), exhibiting fluorescence in the green (left) and red (right) spectrum. Grey represents the silver atoms, green and red colours represent silver cations. Figure from Taccone *et al.* (2018), *Physical Chemistry Chemical Physics* ([45]. (Image reprinted with permission of the rights holder, Royal Society of Chemistry).

Obtaining structural information of Ag-DNA is challenging. Many conformational states are very short-lived (milliseconds to seconds), thus only long-lived states can be measured. Interestingly, dynamic microstates were found to be intrinsic to the long-lived states [36]. In general, conformational transitions have been associated with optical changes [46, 47], but whether these microstates might influence the fluorescent properties of Ag-DNA, and how, was not determined. The shape of the DNA template is reorganized as silver ions bind to cytosine and guanine bases via nitrogen and oxygen sites. Hereby, an ion can be shared between these attachment sites within one base [36]. Contrary, in polyadenine and polythymine, silver ions were found to primarily affect sugar geometries [36].

Generally, single stranded DNA is employed as a template as it is more flexible than double stranded DNA and thus enables better silver incorporation. The commonly used template length consists of 12-30 bases [16, 23]. Due to steric constraints, short templates (< 15 bases) often form dimers which frame the silver cluster [34]. Longer templates also permit interstrand binding, like their short counterpart. Additionally, they often form intrastrand products by wrapping around the silver cluster. The shape of the neutral silver cluster core was repeatedly demonstrated to be rod-like [18, 19], whereby peripheral silver ions align along the DNA template. Thereby, their fluorescence properties are similar to larger nanorods, with resonance largely determined by their sizes and aspect ratios.

The optical properties of Ag-DNA were found to be primarily determined by the size and shape of the silver core. Larger clusters can be associated with higher wavelengths, as they permit greater delocalization of electronically excited states [39]. Dark species were found to appear at lower silver number (< 10), but silver content of 10 - 15 occurred both in dark and bright species [39]. Thus, silver content number by itself does not control whether the nanomaterial is fluorescent. Certain dark complexes can still be optically switched to become fluorescent e.g., by adding a guanine-rich template [24].

The total silver content of one cluster generally lies between 10-24 atoms [44], including neutral and positive atoms. A cluster with an equal number of neutral atoms may exhibit different numbers of ions and thus different charges, and vice versa. Analysing over 600 of 10-base templates and assessing past literature, Copp *et al.* (2014) found a domination of specific atom numbers, independent of the DNA length and sequence [44]. Groupings of 4 and 6 neutral atoms suggest enhanced stabilities around these numbers, while displaying wide-ranging numbers of cations. These termed “magic numbers” correspond to emissions at green (540 ± 20 nm) and red (630 ± 30 nm), respectively, producing the “magic colours”.

The fluorescence properties of Ag-DNA after synthesis can be influenced by factors that influence the DNA conformation such as pH and ionic strength. Additionally, silver ion movements can affect the shape of the silver chain modifying its aspect ratio and/or curvature, and consequently shifting the fluorescence spectra [48]. Smaller wavelength differences between Ag-DNA can also emerge from fluctuations in the number or type of bound bases [49]. This shows the incredible tunability of Ag-DNA pre and post synthesis.

Another remarkable feature is the existence of two distinct absorbance bands and only one emission band. Excitation of the nanomaterial can occur directly via the silver cluster or via the DNA bases [45, 50]. The excitation band via the silver cluster can be tuned within 480-750 nm, as discussed above. Contrary, excitation via the DNA bases is fixed at 260-270 nm and is general to all Ag-DNA species. For more information regarding the origin of photoluminescence and photodynamical properties of the DNA-Ag system, see Thyrhaug *et al.* (2017) [51] and Berdakin *et al.* (2016) [52].

1.2.3. AG-DNA IN CELLS

In contrast to the wide range of publications focusing on sensing ions, small molecules, and proteins in solution *in vitro* by Ag-DNA, research published on applications in (liv-

ing) cells is limited [53–55].

Despite the general high photostability, Ag-DNA face challenges in biological applications. Due to the highly negatively charged DNA backbone, repulsion between the membrane and the phosphate groups leads to poor internalization into cells. To circumvent this, methods like microinjection and transfection have been used successfully [56–58]. However, both methods are time-consuming and impact cellular viability. Further, modifications like the attachment of aptamers or cell penetrating peptide to DNA template were shown to achieve targeted cellular binding or cell penetration. For fixed cells, linking antibodies to Ag-DNA resulted in intracellular staining [55]. Another important challenge is the poor chemical stability of Ag-DNA in biological media and cellular environment. High salt concentrations present in physiological buffers or cell culture media usually lead to silver chloride precipitation and thus destruction of the nanoclusters. Additionally, nucleases within cells can digest the DNA template.

Choi *et al.* (2011) improved Ag-DNA stability by modifying several factors: increasing the base/silver ion ratio during synthesis, elongate and adapt the DNA template, and performing the synthesis directly in physiological buffer or cell culture media [55]. Another group succeeded to improve the stability by forming complexes comprised of Ag-DNA and polycationic electrolytes [57]. As there is high interest in simple and label-free bioimaging probes, the application of Ag-DNA will likely expand in this field when these fascinating nanomaterials are better understood.

In chapter 2 we present three different Ag-DNA forms with three different functionalities. Depending on the employed sequences either stable forms are created that can be used for intracellular labeling purposes or more sensitive forms that are useful as intracellular, optical sensors. Furthermore, the inherent cytotoxic effect due to the silver content in Ag-DNA materials, can be tuned and allows the generation of either biocompatible or low to high cytotoxic probes. Considering the growing interest of researchers to measure the biochemical processes and concentrations inside living cells, Ag-DNAs potential as a sensitive intracellular sensor becomes more and more relevant. Next, the tunability of the toxicity is also highly relevant and can become a powerful tool in cancer research and treatment.

1.3. POLYMERSOMES

1.3.1. POLYMERSOMES BACKGROUND AND PROPERTIES

The organisation and function of a eukaryotic cell is enabled by spatial compartmentalization with lipid membranes. The cell itself is separated from its environment by a double layered lipid membrane, mainly consisting of phospholipids and cholesterol. Likewise, within the cell, biomolecules and ions are confined within subcellular compartments allowing separation of biochemical reactions and degradation processes, as well as intracellular delivery and protection of biomolecules.

Beyond internal vesicles, which are crucial for intracellular function, extracellular vesicles (EVs) exist to coordinate intercellular communication. Practically all biological fluids have been found to contain EVs, including blood, saliva, breast milk, and in the supernatant of cultured cells [59], confirming their importance in cell-cell communication. EVs can efficiently deliver various biomolecules like DNA [60], RNA [61], or

functional proteins [62] to target cells. Hereby, the lipid membrane protects these cargoes from degradation by nucleases and proteases, which are commonly present in the extracellular environment. Currently, EVs are in the focus of attention as they have been demonstrated to participate in important physiological and pathological processes, like the immune response or neuronal degeneration [63, 64]. Due to these exceptional aspects, scientists are highly motivated to use this carrier system deliberately for disease treatment and biomedical research.

Although strategies exist to extract naturally occurring EVs and to load them with exogenous cargoes, these "modified-EVs" suffer from low yield, low purity, and little flexibility [65]. This challenge inspired researchers to create artificial EV counterparts, mimicking the lipid membrane which physically separates the aqueous inner compartment from the surround environment. While EVs have the advantage of full biocompatibility, artificial vesicles (AVs) can be easily synthesized in higher concentrations and modified according to the application requirements.

Liposomes are the best-known AVs and consist of a lipid bilayer of phospholipids, the natural components also integrated in the cellular membrane and vesicles. Their structure allows hydrophobic molecules to be encapsulated in the membrane, and hydrophilic molecules to be loaded into the aqueous lumen. Liposomes have been widely employed in research and as drug carriers, whereby several liposome formulations are FDA-approved [66]. However, liposome surface is strongly affected by interactions with circulating proteins in blood, a phenomenon called opsonization. Thus, liposomes generally display fast clearance due to opsonization by serum proteins and detection by the immune system [67]. Their circulation time can be highly increased by incorporating the polymer polyethylene-glycol (PEG) on the vesicles surface, thus sterically inhibiting electrostatic and hydrophobic interactions with plasma proteins [68]. However, further limitations are low stability and poor flexibility in the synthesis of conventional liposomes.

The main goal of AV development, is to create vehicles which can be loaded with variable cargoes, subsequently protect this cargo from degradation, and efficiently deliver it in a time- and space-controlled way, according to the required question. Thus, high tunability of the carrier vehicle is necessary. A successful approach to fulfill these needs is creating vesicular carriers by using fully synthetic polymers. Discher *et al.* (1999) was the first to implement amphiphilic block copolymers, which chemically resemble cellular phospholipids while displaying increased structural stability and mechanical resistance compared to liposomes [69]. The huge advantage of polymersomes is the high versatility of their physicochemical properties, which allows careful tuning of the physical properties of the AV for any biochemical application.

For the synthesis, one hydrophobic and one hydrophilic polymer are used as a pair, which self-assemble into a bi-layered structure as hydrogen bonds form between the polymers and the surrounding water molecules [70]. The palette of possibble constructions is considerable due to the broad range of available polymers like polyisobutylene-monomethyl polyethylene (PiB) or poly(2-(diisopropylamino)ethyl methacrylate) (PDPA). PEG plays also here an important, due to its stealth properties which grant the carrier with longer bioavailability *in vivo* [71].

1.3.2. POLYMERSOMES IN CELLS

The transport of polymersomes over the cellular membrane is crucial for any therapeutic or imaging application. Intracellular uptake of moieties depends on several factor, and polymersome size plays an important role [72]. Thus, the tuneability of polymersome diameter is highly advantageous and can be controlled via polymer concentration and mixing rate during synthesis [73]. Often the yield contains a broad size distribution, but this polydispersity can be reduced by using standard AV purification techniques like centrifugation or size exclusion chromatography [74].

One of the ways polymersomes can enter cells is via the clathrin-mediated endocytosis. Hereby, particles form contacts with cell surface receptors, initiating the cellular membrane to wrap around and subsequently internalize them [75]. Thereafter, the internalized vesicle (endosome) is actively transported along the cytoskeleton towards the perinuclear area, where the vesicle contents are selected for uptake or secretion. To achieve targeted delivery to particular cell types, polymersome surface is often modified with homing ligands such as aptamers, antibodies, metabolites [76]. Further, controlled cargo release can be attained by creating stimulus-responsive polymersomes which dissociate in response to pH, temperature, redox agents, or light [77]. Due to its synthetic nature, the clearance of polymersomes is often lower than for liposomes, which ensures longer circulation times but can lead to undesired accumulation. Employing biodegradable polymers like polylactide (PLA) and poly(caprolactone) PCL is thus becoming a favourable choice [78].

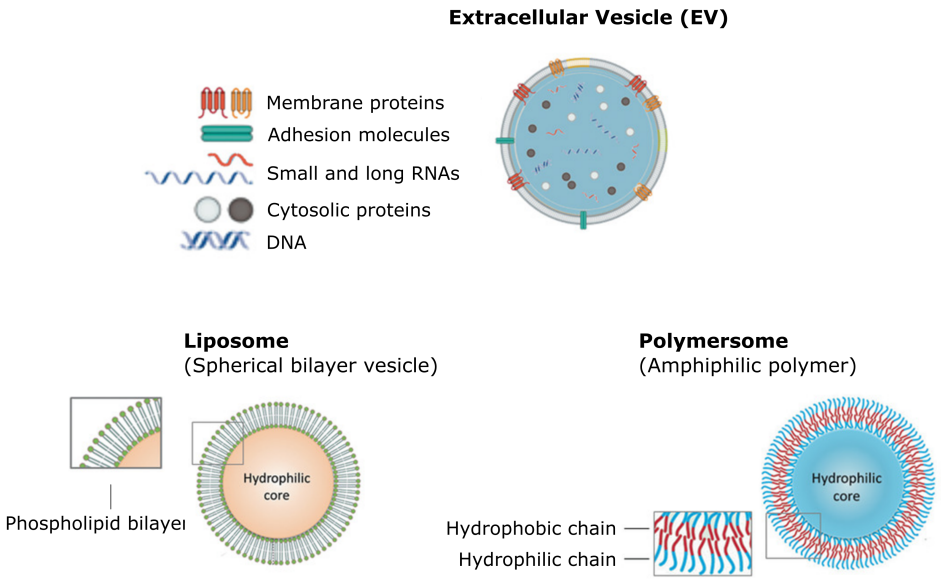

Figure 1.2: Illustration of the membrane compositions of extracellular and artificial vesicles. a) An extracellular vesicle (EV) is comprised of membrane proteins and adhesion molecules, containing various molecules in its aqueous interior. b) A liposome is comprised of a shperical bilayer vesicle with a hydrophilic core. c) The membrane of a polymersome vesicle is built by amphiphilic polymers, containing hydrophobic and hydrophilic chains, and encloses an aqueous interior. Modified figure from Leggio *et al.* (2020), *Advanced Healthcare Materials* ([79]. (Image reprinted and adapted with permission of the rights holder, John Wiley & Sons - Books).

Morphology of polymersomes can also be controlled via the ratio of hydrophilic-to-hydrophobic sections resulting in micelles, ellipsoids, vesicles, or tubular structures. Spherical polymersomes are more common being the thermodynamically most stable structure. Further, the choice of polymer as well as the hydrophilic-to-hydrophobic ratio determine additional physicochemical properties such as the permeability, thickness, rigidity, and surface charge of the membrane [70]. All features influence polymersome function as well as cellular uptake and thus enable adaptability of the carrier to the question of research.

Polymersomes high mechanical and chemical stability as well as their capability to encapsulate molecules makes them interesting systems for *in vivo* fluorescence imaging in cells, tissue, or small animals. As carriers of imaging probes, they serve to improve fluorophore limitations like low tissue penetration, short half-life circulation and fast photo-bleaching. Due to their capacity to entrap large volumes of moieties in their inner aqueous compartment as well as their membrane, high local concentration of fluorophores can be achieved yielding robust fluorescent signal. Combining pH-sensitivity and fluorophore-loading, Massignani *et al.* (2010) achieved efficient endosomal escape and subsequent cytosolic labelling of living cells [80]. By comparing Rhodamine encap-

sulated in a polymersome to not encapsulated CellTracker^{TM} dye, the former was found to be longer fluorescent (up to 14 days) and non-toxic, in contrary to the pure dye, confirming the advantages of employing polymersomes.

Fluorescent polymersomes are widely employed for imaging purposes in living cells or whole organisms like the zebrafish [81, 82]. Further, they are highly interesting for biomedical applications focusing on targeted drug-delivery and tumor-imaging [70]. However, studies are lacking that analyse the dynamics of polymersome uptake, their potential as long-term imaging probes, as well as their degradation in cells. Investigating these processes is important and would provide more insights for research and biomedical application of polymersomes.

In chapter 3 the capacity of polymersomes to entrap an aqueous and a lipophilic dye simultaneously and their ability to preserve the fluorescence of these dyes longer was used. Here, dual-labeled polymersomes were employed for live-cell imaging. By labelling the membrane as well as the interior of the nanovesicle, a great tool was created that allowed to observe the integrity of the polymersomes inside living cells and follow them over days, with hardly impacting the cellular health state. We observed a fast uptake of the dual-labeled polymersomes and could confirm their stable fluorescence over an imaging period of 86 h. Further, the particles were divided on average 50-50 among daughter cells, thus facilitating tracking of all cells equally while their proliferate. At day 7, we observed a strong decrease in fluorescence intensity, whereby the membrane dye showed a higher signal than the encapsulated dye, suggesting a rupture of numerous polymersomes. This highlights the practicality of using dual-labeled vesicles for imaging as a clear indicator of their state. Our results showed that the employed polymersomes are excellent candidates for *in vivo* imaging application for observation periods of up to 3 days.

1.4. NEURAL PROGENITOR CELLS

1.4.1. BACKGROUND INFORMATION

Neurogenesis is a complex process whereby new neural cells are generated. It takes place through proliferation, migration, and eventual differentiation of neural progenitor cells (NPCs) into mature neurons and glial cells. Neurogenesis mainly occurs during embryonic and early postnatal development but is also known to prevail throughout adulthood [83, 84]. In the adult brain, NPCs reside within specialized neurogenic niches which provide the necessary environment to support their maintenance and development of NPCs [85]. NPCs are characterized based on their location in the brain, gene expression profile, temporal distribution, and morphology [86]. Since the research in this thesis mainly focuses on the general mobility and interaction characteristics of NPCs, the complex organization of these niches as well as the terminology of NPC subtypes will not be elaborated on. For more information on this topic see Obernier and Alvarez-Buylla (2019) [85] and Martinze-Cerdeno *et al.* (2018) [86].

Between species, major differences exist regarding the spatial regions in which neurogenesis occurs as well as the extent to which neurogenesis happens [87]. In rodents, the two prominent niches are the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone in the dentate gyrus (SGZ) of the hippocampus. In humans, the

SGZ and the striatum are most active [88, 89].

Neural progenitors in the SGZ generate new excitatory neurons for the dentate gyrus, which are important for learning, pattern recognition, and memory [90, 91]. In the SVZ, NPCs are destined to migrate long distances (3-8 mm in mice) to the olfactory bulb (OB) and differentiate into olfactory interneurons [92]. These are important for fine odour discrimination as well as odour-reward association [93, 94].

The pathway connecting the SVZ and OB is called the rostral migratory stream (RMS) (Figure 1.3. Here, NPCs migrate in chains using blood-vessels and glia cells as guiding structures [95, 96]. Interestingly, in case of an injury, the traveling NPCs can exit the RMS, or migrate directly from the SVZ, towards the injury side following the inflammatory signal [97, 98]. Once arrived at their destination, NPCs can generate functional neurons and integrate into the existing network [99, 100], showing regenerative capacity of the adult brain. Since the heterogeneity of neural subtypes in the CNS is huge, an extensive variety of cell types must be generated for regeneration.

1.4.2. NPCS FOR CENTRAL NERVOUS SYSTEM REGENERATION

NPCs are an exceptional tool here as their multipotency enables them to differentiate into different neuronal and glial cells, depending on the environmental cues. Besides this direct regenerative mechanism, NPCs secrete neurotrophic factors including BDNF, NGF, and VEGF, which support the survival and function of damaged tissue [101, 102]. For this reason, the “secretome” of NPCs has been in focus of research and has been shown to improve the condition of neurodegenerative diseases in animal models [103, 104]. Neurodegenerative diseases like Parkinson’s disease (PD), Alzheimer’s disease (AD), or amyotrophic lateral sclerosis (ALS), as well as injuries like stroke and spinal cord damages, affect millions of people worldwide without the possibility to arrest its progress or cure its state. Considering the current lack of efficient treatments for central nervous system (CNS) injuries and degenerative disease, the regenerative ability of NPC is highly interesting. Despite the natural ability of NPCs to regenerate, the innate response is by far not sufficient to recover the damages caused by physical injuries or neurological disorders [105–107].

Currently, two promising approaches exist which focus on harnessing the full regenerative potential of NPCs. The first way is the mobilization and recruitment of endogenous NPCs. Utilizing internal NPCs has the advantage of avoiding immunocompatibility problems [108]. Mobilization means achieving a higher release of NPCs from their niche, and recruitment means attracting the cells to a specific location. For both goals, biochemicals can be employed. For NPC mobilization, Leukemia inhibitory factor (LIF) can be used, as it plays an important role in proliferation and fate regulation [109, 110]. To recruit NPCs to the injury location, factors like stromal cell-derived factor-1 (SDF-1) and hepatocyte growth factor (HGF) have been shown to successfully act as chemoattractants [111, 112]. The second way is the injection of exogenous NPCs which have been differentiated *in vitro* or isolated from another source. Transplantation of exogenous NPCs into animal models of various neurodegenerative diseases has been shown to result in functional improvements [113, 114]. The benefits of this method include the possibility to control various factors, e.g., by genetically modifying cells to overexpress

neurotrophic factors. Current challenges with this technique include well-characterized cells, height of dose numbers, low viability upon transplantation, as well as sufficient migration, differentiation, and network integration at the impaired location [115].

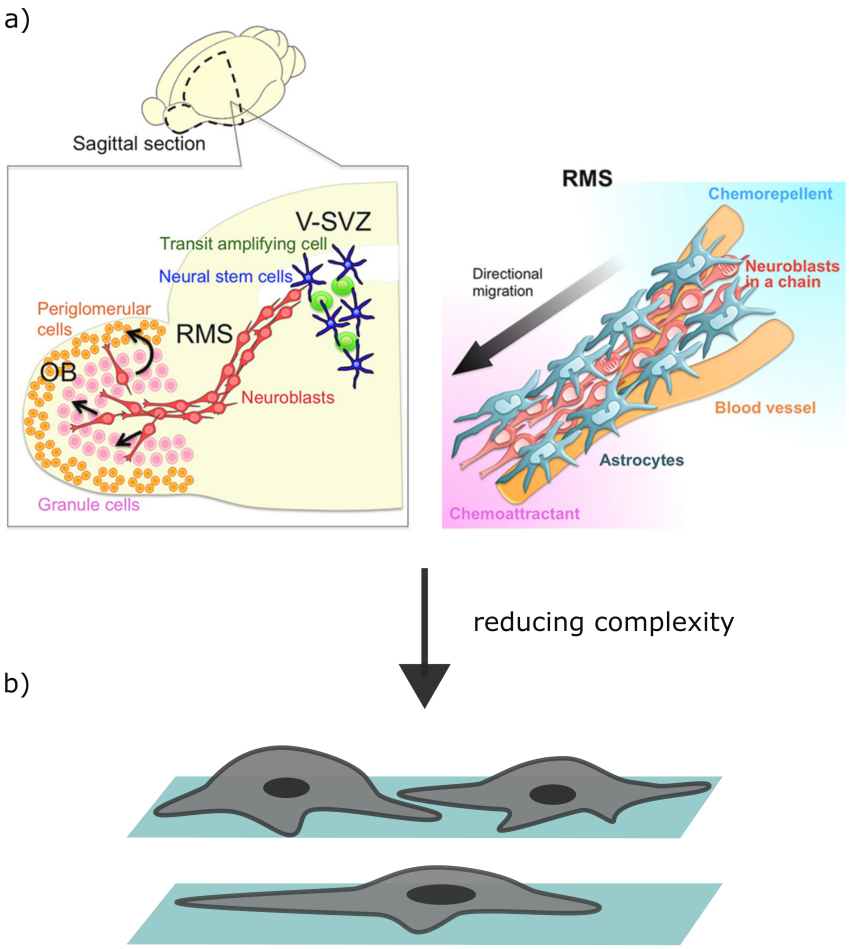

Figure 1.3: Illustration of RMS and V-SVZ localization and composition. a) Overview of the migration of neuroblasts within the RMS, starting in the V-SVZ and moving towards the OB. Overview of the situation of an injury and the migration of neuroblasts from the RMS and V-SVZ towards the lesion site. Figure modified from Kaneko *et al.* (2017), ([116]. (Image reprinted and adapted with permission of the rights holder, John Wiley & Sons - Books)

For therapeutic effects of NPC treatments, it is crucial that high numbers of cells arrive at specific locations. Direct injection is not always possible, especially when the damage is spread out throughout the brain as in neurodegenerative diseases. Thus, it is essential to understand the migration mechanism of NPCs and the influence of the environment on their motion capability. Available studies on NPC migration mainly focus on their progressive movement within days or weeks, and the final arrival at the side of inflammation [117–119]. Although these results give valuable insights into the migratory capacity of NPCs, data on the fundamental migratory behaviour on the timescale of minutes and hours is missing.

Despite the success of complex *in vivo* studies, *in vitro* experiments can still give important information as they provide a clean and fully controllable environment. Most *in vitro* studies on NPC focus on their lineage progression [120, 121], as their commitment to a differentiated state is still an important field to understand, or they look only at the broad migration distances [122]. However, hardly any studies analyse the fundamental migratory behaviour of NPCs. Since multiple factors influence the safe journey of migrating NPCs, collecting *in vitro* migration data on a single-cell level will enhance understanding of their innate characteristics and ultimately contribute to advancement in clinical applications. Multiple possibilities exist to reduce the complexity of an *in vivo* system but still emulate the different factors that cells encounter while migrating. By using patterning techniques to print lines and other geometries on 2D surfaces, minimalistic systems can be created that mimic the scaffold of blood vessels and astrocytes along which NPCs migration *in vivo*. To simulate the pores and tight openings that NPCs need to squeeze through on their journey, 3D channels with confinements present a great *in vitro* analysis system. Both systems are employed in chapter 5.

Chapter 5 focuses on the biophysical basics of NPCs with and without physical constrictions using patterned surfaces. In experiments we observed that NPC motility can be described by the mathematical persistent-random motion (PRW) model, whereby cells move with persistence on long time scale but show random motion on long time scale. Constricting NPCs to line patterns did not influence their velocity but did reduce the persistence time. Interestingly, by observing the cells visually on lines and more complex geometries, we detected several behavioral patterns highlighting how these cells interact with each other and explore their environment. Lastly, NPCs readily migrated through narrow 3D channels and squeezed through tight pores with a smaller size than their nucleus. The preliminary observations of NPC made on the various *in vitro* environments contribute to our understanding of their inherent behaviour and provide tools for further investigations.

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