A novel polymer drug conjugate for the sustained release of ganciclovir

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**A NOVEL POLYMER DRUG CONJUGATE FOR THE
SUSTAINED RELEASE OF GANCICLOVIR**

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**Interdisciplinary Graduate School
NTU-Northwestern Institute for Nanomedicine**

2019

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**INTERDISCIPLINARY GRADUATE SCHOOL
NTU-Northwestern Institute for Nanomedicine**

A thesis submitted to the Nanyang Technological
University in partial fulfillment of the requirement
for the degree of Doctor of Philosophy

2019

Statement of Originality

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Authorship Attribution Statement

The thesis material is not published yet in any peer reviewed journal.

The contributions to the project work are as follows:

- Prof. Subbu S. Venkatraman has supervised the project work and guided the direction of the project.
- Dr. Rupesh Agrawal has mentored the project work.
- Prof. Andrew Lee has co-supervised the project work.
- I did all the experiments and characterization for my project work.
- I performed all the laboratory work at the School of Material Science and Engineering, Singapore and analysed the data myself.

22.08.2019

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Abbreviations

2-(N-morpholino)ethanesulfonic acid	MES
Acetonitrile	ACN
Acquired immune deficiency syndrome	AIDS
Acylated valganciclovir	Ac-ValG
Cytomegalovirus	CMV
Dibasic sodium phosphate	NaH ₂ PO ₄
Ganciclovir	Gan
High performance liquid chromatography	HPLC
Human immunodeficiency viruses	HIV
Hydrochloric acid	HCl
Methanol	MeOH
Monobasic sodium phosphate	Na ₂ HPO ₄
N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide	EDC
N-hydroxysuccinimide	NHS
Ocular manifestation of cytomegalovirus	CMV retinitis
Phosphate buffered saline pH 7.4	PBS
Phosphoric acid	H ₃ PO ₄
Poly(lactide-co-glycolide)	PLGA
Polyacrylic acid	PAA
Polyacrylic acid - L-valine -ganciclovir conjugate	PAA-ValG
Sodium azide	NaN ₃
Sodium chloride	NaCl
Sodium hydroxide	NaOH
Tan Tock Seng Hospital	TTSH
Triethylamine	TEA
Trifluoroacetic acid	TFA
Valganciclovir hydrochloride	ValG*HCl

Abstract

Ocular manifestation of cytomegalovirus (CMV retinitis) remains the most common AIDS-related ocular complication which occurs in 40% of HIV patients and causes loss of vision or blindness [1]. Intravitreal injection of ganciclovir (an inhibitor of the viral DNA polymerase [2]) is the current gold standard to treat CMV retinitis. Since there is no sustained release drug delivery system currently available [3], patients suffer from side effects of the frequent intravitreal injections such as retinal detachment and hemorrhage [4, 5].

This study aims to explore a ganciclovir-loaded polymer drug conjugate for the long-term sustained release for the treatment of CMV retinitis. Considering the high drug loading, is necessary to be able to reduce the number of intravitreal injections, a PAA-ValG conjugate comprising polyacrylic acid (PAA) as polymer backbone, L-valine as linker and ganciclovir as drug, were chosen. The conjugate was synthesized by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) coupling of valganciclovir (L-valine ester prodrug of ganciclovir) to polyacrylic acid in aqueous buffer solution. Multiple conjugates of different molecular weights were synthesized in different stoichiometric ratios of reactants. HPLC analysis was used to quantify ganciclovir, as well as valganciclovir and impurities or degradation products from the extracted (free drug) or hydrolyzed (assay) reaction products. It was shown that the 10 batches of higher stoichiometric ratios (1, 1.5 or 2 mol coupling reagent per mol COOH from PAA) contained 42.0% ganciclovir (w/w) on average, which were higher than all other studies in literature reported [6-12]. An effect of the chosen PAA (1.2, 8, 15 and 30 kDa) as well as the stoichiometric ratio was not observed, hence the standard deviation for the drug loading (assay) over all 10 samples was only 1.1%. This constant conjugation of 26.6% of all COOH groups from PAA was expected to be caused by steric hindrance. The content of free drug was < 0.5% of the total drug and unknown ingredients were < 0.2% for all mentioned samples, which showed that the purification process worked. Analysis of the samples after 4 months of storage showed no significant release of drug (free drug) or increase in degradation products, which showed the high storage stability of the conjugates. The *in vitro* release of ganciclovir from PAA-ValG was performed in a release medium which has all relevant properties of vitreous humor, with the exception of biological ingredients. A long-term sustained release over a period of more than 1 year was observed, which was much longer than

all other ganciclovir-releasing systems reported [6-12]. The release rates were noticed to have decreased slightly over time, from 8.2% per month in the 1st month, to 2.2% per month in the 12th month on average. *Ex vivo* release experiments in pig-vitreous showed a similar release in the solid state and approximately 8 times faster release in the soluble state of PAA-ValG, in comparison to the *in vitro* release testing. Similar behavior of polymer drug conjugates in biological fluids was also confirmed by D'Souza et. al., since release enhancing enzymes like esterase can enter polymer drug conjugates in the soluble state, but not in the solid state [13]. Since PAA-ValG was observed to change from the solid to the soluble state during release, depending on the molecular weight, concentration and amount released, the *in vivo* release of PAA-ValG is expected to be faster, but also more constant than the observed *in vitro* release profile indicated. In the best case, one injection of around 11 mg PAA-ValG (depending on the exact drug content), which is equivalent to 4.5 mg ganciclovir will be sufficient for 6 to 8 months of treatment, as it was in the later discontinued ganciclovir-releasing implant Vitrasert [3, 14], but with less side effects, due to injectability. Considering the high drug loading, purity, stability and the long-term sustained release, as well as the predicted properties from literature, PAA-ValG seems to be an excellent candidate for *in vivo* studies, with the goal to control cytomegalovirus retinitis with fewer intravitreal injections as per the current treatment. To complete the work, the release mechanism of ganciclovir from PAA-ValG in a simulated physiological ocular environment was studied. Acylated valganciclovir (Ac-ValG) was synthesized to study the ester hydrolysis without the effects of the polymer, since its chemical structure next to relevant ester bond is similar. The reaction constants for the pseudo-first-order release rate of Ac-ValG were determined and found to be pH dependent and increasing linearly with the hydroxide ion concentration in the release medium. Furthermore the penetration of hydrolyzing agent into the polymer (degree of hydration) was studied by tablet swelling experiments, as previously performed by Pitt and Shah [15] and found to be increasing with time and pH. Also the decrease in the microclimate pH during release, as it is known from other biodegradable polymers [16] was studied via titration and found to be stronger at high pH values, and the beginning of the release. The combination of reaction constant, microclimate pH, concentration of cleavable drug, and the degree of hydration leads to a formula which describes the release rate accurately.

1. Introduction

1.1. Background

Depending on the standard of living, approximately 30% to 90% of the world population is infected by cytomegalovirus (CMV) [17]. In healthy beings, the virus count is actively kept under control by the immune system, hence chronic infection of the host usually remains subclinical. Nevertheless, in patients with immune deficient conditions (e.g. HIV patients), reactivated dormant cytomegalovirus can pose a severe threat to the patient's life where the virus would reach the eyes through blood circulation and can cause blindness in the infected individual due to CMV retinitis [1]. At the moment, there is no sustained drug delivery system for ganciclovir (the current gold standard for the treatment of CMV) available [3], which could reduce the treatment frequency and side effects related to intravitreal injections, intravenous injections and orally administered ganciclovir [4, 5, 18].

Of the mentioned drug administration types, intravitreal injected ganciclovir has the highest need for a sustained releasing drug delivery system, as frequent intravitreal injections are known to cause numerous complications including retinal detachment and hemorrhage [4, 5].

1.2. Problem statement

The options for the long-term sustained release of hydrophilic drugs such as ganciclovir are very limited, especially when the drug loading has to be high, as is the case for ocular applications. So far, it is mainly implants which have been discovered to be suitable for this purpose, such as the intravitreal implant **Vitrasert**, which is able to deliver ganciclovir over 6 to 8 months [14], but was discontinued [3]. The invasive implantation, as well as the removal of non-biodegradable implants are an issue [19].

Hence, it would be desirable to have an injectable drug delivery system for a long-term sustained release of ganciclovir to treat CMV retinitis.

1.3. Hypothesis and Objectives

The following hypotheses and objectives were studied in this work:

1.3.1. Hypothesis 1

A polymer drug conjugate consisting of a polyacrylic acid backbone, L-valine as linker and ganciclovir as drug can be successfully synthesized by coupling valganciclovir (L-valine ester prodrug of ganciclovir) to polyacrylic acid, using aqueous solutions for reaction and work-up. A pure product named PAA-ValG with high drug loading and storage stability could be obtained by this synthesis method.

1.3.2. Objectives 1

- Synthesis of PAA-ValG, using PAA of different molecular weights and different stoichiometric ratios of reactants.
- Confirmation of the proposed chemical structure, using NMR spectroscopy.
- Quantification of conjugated ganciclovir, non-conjugated ganciclovir, impurities and degradation products, using HPLC analysis.
- Stability testing of PAA-ValG.

1.3.3. Hypothesis 2

The *in vitro* and *ex vivo* release of ganciclovir, as well as predicted properties of PAA-ValG from literature, such as toxicity, pharmacological activity and clearance from the vitreous will make PAA-ValG a good candidate for *in vivo* studies, with the goal to control cytomegalovirus retinitis (CMV) with fewer intravitreal injections as in the current treatment.

1.3.4. Objectives 2

- *In vitro* release testing of PAA-ValG in simulated vitreous fluid.
- *Ex vivo* release testing of PAA-ValG in pig vitreous.
- Prediction of toxicity, pharmacological activity and clearance.

1.3.5. Hypothesis 3

The release of ganciclovir from PAA-ValG in a simulated physiological ocular environment is not following a pseudo-first-order rate, as ganciclovir esters of similar structures do, since the polymer is affecting the hydration of the ester bond (degree of hydration), as well as its surrounding (microclimate) pH value.

1.3.6. Objectives 3

- Synthesis of acylated valganciclovir, by coupling acetic acid to valganciclovir.
- Determination of the reaction constants for the ester hydrolysis of valganciclovir and acylated valganciclovir at different pH values.
- Determination of the degree of hydration of PAA-ValG, using tablet swelling.
- Determination of pH shift in microclimate of PAA-ValG during release.
- Combination of the determined values with the release data of PAA-ValG to validate the proposed mechanism.

2. Literature Review

2.1. Cytomegalovirus (CMV)

Cytomegalovirus is a dsDNA virus of Herpesviridae family with a protein enveloping outside. As a member of the Herpesviridae family, it is persistent in the host, down regulating MHC Class 1 molecules in infected salivary gland tissue and evading natural killer cells [20-22]. CMV migrates from person to person through transfer of body fluids and organ transplant and can cause retinitis, hepatitis [23], colitis [24], pneumonia [25], or encephalitis [26] in the case of immunocompromised individuals. In patients with immune deficient conditions, reactivated dormant cytomegalovirus can pose severe threat to the patient's lives. Amongst all other symptoms, ocular manifestation of cytomegalovirus (CMV retinitis, figure 2.1.) remains the most common AIDS related ocular complication which occurs in 40% of HIV patients [27].

Figure 2.1.: Cytomegalovirus retinitis [28]

CMV retinitis appears as thick necrosis in retinal region and edema/exudative attachment [29, 30]. CMV retinitis begins in peripheral region of retina and advances towards posterior pole at 24 mm/day [31].

Complete blindness or loss of vision are caused by the following events [32]:

1. Retinal necrosis
2. Retinal detachment
3. Optic nerve necrosis
4. Cataract formation
5. Epiretinal membrane

Clinical diagnosis CMV retinitis is dependent on the presence of lesions. However, CMV polymerase reaction can also be conducted if the diagnosis is unclear. Fundus photography is another technique to screen CMV retinitis and enable telemedicine in regions which does not have access to diagnostic facilities [33, 34].

2.2. Conventional treatments of CMV retinitis

Conventional treatments of CMV retinitis which includes ganciclovir, can help to slow down the progression of the disease by acting as an inhibitor of the viral DNA polymerase [2]. However, the hydrophilic nature of the drug results in poor oral bioavailability [35]. Hence, ganciclovir requires intravitreal injections, intravenous injections or ocular implants. High drug concentrations are needed for the intravenous treatment of CMV retinitis which could lead to adverse reactions [18], while intravitreal injections are known to cause numerous complications including retinal detachment and hemorrhage [4, 5]. Ganciclovir-resistance is also possible due to mutations in the kinase bioactivating enzyme UL97 of CMV [36, 37]. Most physicians recommend systemic treatment in addition to intravitreal injections [4], to prevent infection of the other eye. The development of valganciclovir, a L-valyl ester prodrug of ganciclovir, shows enhancement in bioavailability [38] due to it being transported via the peptide transporter PEPT1 [39], hence allowing it to be taken orally. Nonetheless, since both drugs require either systemic treatment (oral) or intravitreal injections, research has been focused on developing new systems for the localized delivery of these drugs.

Figure 2.2.: Ganciclovir [40]

A very well-established treatment of CMV retinitis is performed by the clinician (ophthalmologist) who mentored this project, Dr. Rupesh Agrawal from the Eye Centre of Tan Tock Seng Hospital in Singapore.

It consists of intravitreal injections of ganciclovir in 3 phases:

1. In the **introduction** phase, 2 mg of ganciclovir in 0.04 ml volume are injected twice weekly, over a duration of 4 weeks.
2. In the **high dose maintenance** phase, 2 mg of ganciclovir in 0.04 ml volume are injected once weekly, over a duration of 4 weeks.
3. In the **low dose maintenance** phase, 1 mg of ganciclovir in 0.02 ml volume is injected once weekly, until the restoration of health.

Even though the treatment of CMV retinitis with ganciclovir is overall successful, there are still side effects due to the high dosage and the frequent or invasive administration. So far, no sufficient controlled release of ganciclovir from a drug delivery system has been reported. This leaves many possibilities for further research in this field, to improve the current treatment of CMV retinitis.

2.3. Sites of ocular drug delivery / ocular ganciclovir delivery

There are various sites for ocular drug delivery, as shown in the following figure:

Figure 2.3.: Anatomy of the eye with routes for administration of drug delivery [41]

2.3.1. Topical (as eye drops)

Eye drops are widely used as non-invasive localized form of ocular drug delivery. Despite its widespread use as the simplest form of ocular drug administration, the bioavailability is often less than 5%, due to several layers of ocular tissue barriers which have to be penetrated [42]. Hence, eye drops are mainly used to treat outer parts of the eye [19].

Ganciclovir is available as Ophthalmic Gel 0.15% [3] and has to be applied 3 to 5 times daily (1 drop) for the treatment of acute herpetic keratitis (dendritic ulcers) [43]. The penetration is insufficient for the inner ocular treatment of CMV.

2.3.2. Intravitreal (as injections)

Intravitreal injections are the most direct way of delivering drug into the vitreous humor which has a volume of approximately 4 ml [44]. The drug reaches the retina after diffusing through the vitreous humor and crossing the internal limiting membrane (ILM), a barrier of extracellular matrix proteins [14].

Ganciclovir is available for intravitreal injections [45, 46], dosage as in section 2.2.

2.3.3. Periocular (as injections)

Periocular drug administration includes subconjunctival, sub-tenon, peribulbar and retrobulbar routes [47]. The advantage of periocular injections over intravitreal injections is its ability to deliver drugs sustainably, since the drug has to penetrate from the peripheral region [19]. Also, the possible complications are fewer. For example, retinal detachment may occur when injecting intravitreally [47].

Antiviral intravitreal concentrations of ganciclovir could not be achieved with subconjunctival injections in rabbits [48]. There is no approved form of ganciclovir available for periocular injections [3].

2.3.4. Ocular (as implants)

Implants can be placed in most of the sites for ocular drug delivery e.g. in vitreous chamber, periocular space, or conjunctival sac. Implants provide the advantage of controlled release over long periods of time, but the procedure for implantation and removal of non-biodegradable implants often require surgery where complications could occur. Depending on the potency of the drug which has to be delivered, the size of the implant is often an issue [19].

The ganciclovir eluting intravitreal implant **Vitrasert** was approved for the treatment of CMV retinitis, but later discontinued due to financial reasons [3]. **Vitrasert** delivered ganciclovir over a long period of 6 to 8 months but was non-biodegradable. The mechanism of release was diffusion-controlled through a small opening in a non-permeable membrane [14].

2.3.5. Preferred site for ganciclovir delivery

Considering all possible sites of ocular drug delivery, it seems that ganciclovir can only be applied through intravitreal injections to treat CMV retinitis effectively. This can be done either frequently via intravitreal injections or in the form of a relatively more invasive, but long-lasting sustained release intravitreal implant. Combining the best properties of both would result in an intravitreal injection that is long-lasting.

2.4. Ocular drug delivery / drug delivery systems

Ocular drug delivery research comprises a wide range of systems including biodegradable and non-biodegradable intravitreal implants, microspheres, emulsions, and polymer formulations [49]. The main advantage of drug delivery systems is a controlled and localized drug release which enables stable drug concentrations. This can help mitigate toxicity issues which can be caused by a spike in plasma concentrations and helps reduce the risk of developing resistance. Furthermore, drug delivery systems improve patients' compliance due to long-term release formulations that reduces the frequency of treatment.

2.4.1. Injectable drug delivery systems for ganciclovir

Different approaches for sustained delivery of ganciclovir have been reported, using most of the commonly used injectable drug delivery systems.

Biodegradable microspheres, made from poly (lactide-co-glycolide) (PLGA) of different ratios of lactide to glycolide were reported by Duvvuri et. al. [11], as well as by Xi and Chui [9]. Both research groups reported biphasic release profiles, as they are known from PLGA particles, where an initial slow release rate is followed by a burst release, and then subsequently a slow release rate. Other than the suboptimal release profiles, both groups reported drug loading of 5-7% ganciclovir (w/w), which might be problematic for intravitreal injections, due to limitations in the injectable volume. Xi and Chui also reported two particle batches with a loading of 20% ganciclovir (w/w), where more than 50% of the drug was released in an initial burst release.

Liposomes and Niosomes (liposome-like vesicles from non-ionic surfactants) for the release of ganciclovir were reported. Liposomes from Kajiwarra et. al. showed a release over 3 days, with a significant burst release [7]. Niosomes from Akhter et. al. showed a zero-order release over one day [10].

Albumin nanoparticles, loaded with ganciclovir were reported by Merodio et. al. [8]. A release of 5 days was reported, showing a significant burst release.

Polymer drug conjugates, for the release of ganciclovir have been reported by Sawdon and Peng, using a ganciclovir-PCL-chitosan conjugate with a reported release of 3 days [6] and by Cavallaro et. al., using a Gan-polyaspartamide conjugate with a reported release of 1.5 days [12]. Both research groups reported drug loadings of less than 1% ganciclovir (w/w).

Table 2.1: Reported injectable delivery systems for ganciclovir from literature:

Drug delivery system	Loading (wt. %)	Release reported	Release profile	Source
Biodegradable microspheres	5-7	40 days	biphasic release	[11]
Biodegradable microspheres	5-7	240 days	biphasic release	[9]
Liposomes	5	3 days	significant burst release	[7]
Niosomes	< 20	1 day	zero-order kinetics	[10]
Albumin nanoparticles	3	5 days	significant burst release	[8]
Gan-PCL-chitosan conjugate	< 1	3 days	significant burst release	[6]
Gan-polyaspartamide conjugate	< 1	1.5 days	zero-order kinetics	[12]

Since ganciclovir is a hydrophilic drug, the diffusion-controlled drug delivery systems: **liposomes**, **niosomes** and **albumin nanoparticles** are only showing a short release time, which is not sufficient for a long-term treatment of CMV retinitis.

The same applies for **biodegradable microspheres**, which show a combination of diffusion and erosion-controlled release mechanisms, if the drug loading chosen is too high. On the other hand, low drug loading makes it impossible to apply the sufficient quantity of ganciclovir via intravitreal injections.

Compared to other polymer-based drug delivery systems, **polymer drug conjugates** can carry a higher drug loading (when choosing a simple polymer structure) as well as achieve a sustained drug release without unwanted drug leakage [50].

2.4.2. Preferred drug delivery system for ganciclovir

A polymer drug conjugate seems to be the best option for delivering ganciclovir.

Polymer drug conjugates are products of **nanomedicine** which are mainly used in the treatment of cancer. More than 10 polymer-drug conjugates are currently in clinical development for treatment of various conditions. The site specific and sustained release with lower peak concentrations significantly reduces the damage to healthy cells [51]. Since high drug concentrations of ganciclovir might also cause adverse reactions [18], especially in the peak concentration after administration, a polymer-drug conjugate seems to be a good approach for the controlled delivery of ganciclovir.

2.5. Design of polymer drug conjugates

Polymer drug conjugates usually consist of a polymer backbone and a drug which are combined with a linker. Homing devices for site specific drug release as well as solubility increasing groups can be added optional:

Figure 2.4.: Schematics of polymer-drug conjugate

2.5.1. Polymer backbone

When it comes to the design of a polymer drug conjugate, the choice of the polymer backbone is crucial. The most commonly used polymers for polymer drug conjugates are poly(N-(2-hydroxypropyl) methacrylamide), poly(ethylene glycol) copolymers, and polyglutamic acid. [50].

Polyacrylic acid was chosen as the polymer backbone for the conjugate, because it has more carboxylic acid groups per gram of polymer than most of the commonly used negatively charged polymers. This is important for the preparation of polymer drug conjugates with high drug loading, since only limited quantities or volumes of formulations can be applied to the eye. Further advantages include its biocompatibility, mucoadhesive properties and that it has already been used in ocular pharmaceutical formulations.

Although polyacrylic acid is non-biodegradable, it is however biocompatible, which means that the polymer stays unchanged inside the human body, until it is cleared out. The decision against a biodegradable polymer such as polyglutamic acid was actively made to prevent the polymer backbone to degrade faster than the drug is released, especially since a release over many months is desired.

2.5.2. Labile bond

The bond between the polymer and drug in the polymer drug conjugate plays a vital role and has a significant impact on the drug release kinetics. Esters are the most preferred bonds over carbamate, carbonate, and amides for controlled drug release at physiological pH. The bond can undergo enzymatic or non-enzymatic hydrolysis for the release of drug from polymer drug conjugate. Non-enzymatic hydrolysis is more preferred over enzymatic due to limited diffusivity of enzymes in heterogeneous polymer-drug conjugate systems. Heterogeneous systems involve polymeric hydrogels and polymeric micelles [50].

Types of labile bond used in polymer-drug conjugate systems:

- a) Esters
- b) Carbonate esters
- c) Carbamate esters
- d) C=N linkages
- e) Amides

Hence, the design of the labile bond plays a vital role in designing of polymer drug conjugates for drug delivery due to dependency of drug release rate on hydrolysis kinetics of labile bond.

In this dissertation, the ester bond was chosen as labile bond for designing a ganciclovir releasing polymer drug conjugate.

2.5.3. Spacer

Since the drug has to be released from the polymer, steric hindrance by the backbone to chemical or enzymatic hydrolysis of the labile bond might pose a problem. Due to this reason, a spacer (also called linker) is commonly applied.

L-valine was chosen as the spacer between the polymer backbone and drug. The choice for L-valine among the many amino acids which are commonly used as linkers in polymer drug conjugates was mainly made, due to the fact that the L-valine ester of ganciclovir (valganciclovir) is an approved pro-drug of ganciclovir. This makes it easier to predict the effects of the drug on the human body, in the event that the drug and linker are released, as compared to instances where only the drug is released. Intravitreal injections of 0.2 to 0.4 mg valganciclovir twice weekly are possible [52].

2.5.4. Homing device

Homing devices, also called targeting moiety, are used for the targeted drug release of polymer drug conjugates. They are mainly applied in cancer treatment, when the polymer drug conjugate is applied via intravenous injections/ infusion, but the release of the cytotoxic drugs should mainly happen after the conjugate has reached and bound itself to cancer cells.

Homing devices are not necessary for this planned ganciclovir releasing polymer drug conjugates, since they will be directly injected at the site of action via intravitreal injections.

2.5.5. Solubilizer

Solubilizer are highly hydrophilic groups, which are added to increase the solubility of polymer drug conjugates, should the properties of the system require it.

A solubilizer is not necessary for a ganciclovir loaded conjugate system here, since the solubility prior to injection can be controlled via the pH level and this usually done when injecting ganciclovir as sodium salt. After injection, a low solubility is preferred to reduce the clearance of the conjugate until it is fully released.

2.5.6. Molecular weight

Molecular weight is of great importance while designing the polymer drug conjugate due to its control over bioavailability and residence time inside the body. Polymer drug conjugates of high molecular weight have a longer residence time in comparison to those of a low molecular weight [53, 54]. Even though the polymer with high molecular weight is preferred so that the entirety of the drug can be released at the site of action, however, conjugates with molecular weight (hydrodynamic volume) that is too high will prevent the remaining polymer backbone from getting cleared out of the body. This might cause problems, especially when polymer backbones are non-biodegradable like PAA.

The exact threshold for the clearance of polymers from the vitreous, as well the following renal clearance from the bloodstream are dependent on the hydrodynamic radius which is dependent on the chemical structure and the molecular weight of the polymer [13].

Many different values have been reported e.g. D'Souza et. al. reported a renal threshold of 45 Å, equivalent to 45 kDa HPMA, 30 kDa PEG or 40 kDa Dextran [13], whereas Bertrand et. al. reported a renal cutoff of 29 kDa for Dextran [51] and Kostkova et. al. reported a renal cutoff of 50-70 kDa for HPMA [55].

Amo et. al. studied the effect of the molecular weight (molecular radius) on the clearance of dextran from vitreous, where a decrease of the clearance rate with increasing in molecular weight was shown:

Figure 2.5.: Effect of molecular weight on the clearance from vitreous [53].

Considering the variety of reported values for the clearance from vitreous as well as from the blood stream via renal clearance, it was concluded that the maximum molecular weight for a PAA-based polymer drug conjugate will have to be determined by *in vivo* experiments.

The range of 8 to 30 kDa for the PAA-backbone, with additional increase of the MW due to L-valine linker and the polydispersity of the used polymer would be a good range for the development of ganciclovir releasing polymer drug conjugates for intravitreal application. (refer section 5.3.3)

2.6. Drug release from polymer drug conjugates

As discussed in Section 2.5 about the polymer drug conjugate design, the hydrolysis of the bond present between the spacer and drug is rate-limiting for the drug release; such systems where the spacer is present between the drug and polymer are described as 'tripartite'. The drug can also be directly bonded to the polymer without a spacer and the hydrolysis of drug-polymer bond is the rate-limiting factor for drug release. This is known as a 'bipartite' system. Tripartite systems are the best preferred due to the freedom on bond selection. The different types of bonds have been listed in section 2.5.2. Different types of bonds have different hydrolysis kinetics and release rate due to their interdependency.

2.6.1. Non-enzymatic hydrolytic release

Hydrolysis of the ester bond present in the polymer-drug conjugate can be carried out under physiological conditions. However, labile bonds such as carbonate, carbamate and amides require enzymatic hydrolysis. In heterogeneous systems like hydrogels and polymeric micelles, non-enzymatic hydrolysis is the most relevant due to limited diffusion of enzymes.

Rate of hydrolysis of ester linkages is dependent on the following factors:

- A) Electron withdrawing groups near reaction center – Electron withdrawing groups present near reaction center would enhance the rate of hydrolysis, whereas the electron donating groups would decrease the rate of hydrolysis [13].
- B) Hydrophobic / hydrophilic environment – The hydration of polymer drug conjugate is one of the most influential factors which plays a vital role and has a significant influence on rate of ester hydrolysis. Hydrophobic groups present near the reaction center would decrease the degree of hydration, leading to an eventual decrease in the rate of hydrolysis. However, it has been observed that the presence of hydrophilic groups may instead enhance the rate of hydrolysis in esters. Ajit et al. has proposed that presence of hydrophilic groups near the reaction center would enhance the local dielectric constant and the rate of hydrolysis [13]. Studies by Pitt and Shah have demonstrated the effect of hydrophilic and hydrophobic groups on the degree of hydrolysis. Hydrogels of copolymer 2-p-nitrobenzoxyethyl methacrylate (NBEMA) and methoxydiethoxyethyl methacrylate (MDEEMA) with different proportion have decreased swelling from 62% to 32% with an increase in proportion of hydrophobic NBEMA from 5 to 20 mol%. Hydrolysis follows first

order kinetics at low NBEMA proportion and almost zero order kinetics at higher proportion [15]. In another study, the water-soluble polymer drug conjugate was soluble at lower critical solution temperature and was insoluble at temperature higher than the lower critical solution temperature (LCST). Hydrolysis of the drug from the conjugate had increased the LCST temperature and the solubility of polymer-drug conjugate increase with hydrolysis of drug. The increase in hydration with the hydrolysis of ester linkages has increased the reactivity of the ester hydrolysis and had ended in following zero order kinetics [56].

- C) Steric hindrance – Steric hindrance is another most important limiting factor for the ester hydrolysis. Bipartite polymer-drug system was the most common conjugate used, where the steric hindrance has a significant effect over the hydrolysis of ester linkages. However, the presence of spacer between the polymer and drug would overcome the steric hindrance and would improve the hydrolysis. Studies reported by Larsen and coworkers have studied similar systems. In their work, they observed that the incorporation of space between polymer drug conjugate of dextran-naproxen has increased the half-life of naproxen from 4 h to 180 h in conjugates with spacer [57]. Similar study by Tang et.al. has demonstrated the increase in release rate of norethindrone from norethindrone-poly- α , β -(hydroxyalkyl)-DL-aspartamide conjugates [57].

2.6.2. Enzymatic release

Enzymatic hydrolytic release of drugs from polymer drug conjugates has been carried out for soluble conjugates. Carboxylic esterase present in body fluids has the potential to accomplish hydrolysis in polymer-drug conjugates [12]. Similar to non-enzymatic hydrolysis, enzymatic hydrolysis is also dependent on steric hindrance. Studies reported by Larsen and team have demonstrated lack of hydrolysis in the case of benzoate hydrolysis of dextran. Steric hindrance was reported to be resulted from the dextran backbone. However, the spacer present between drug and polymer has been proposed to be essential for enzymatic hydrolysis. Greenwald and team have studied the PEG based polymer drug tripartite systems and effect of steric hindrance on the hydrolysis of labile bond [58].

The effect of steric hindrance/crowding on the half-life of drug release in rat plasma has been shown in table below:

Compound	#	$t_{1/2}$ (h) Buffer pH 7.4	$t_{1/2}$ (h) Rat Plasma
Daunorubicin·HCl	-	-	-
11Aa	>24	1.9
11Ap	>24	17
11Aβ	>24	0.2
11Ba	>24	21
11Bβ	>24	8
21A	>24	1.1
28A	>24	>24 ^c

Figure 2.6.: Effect of steric hindrance on the half-life in buffer and rat plasma [58]

2.7. Project summary in context of literature

We want to treat CMV retinitis with an injectable intravitreal ganciclovir formulation, comprising of an ester-bond polymer drug conjugate with PAA acting as the polymer backbone, L-valine as the linker, and ganciclovir as the drug, which is expected to have a high drug loading and sustained drug release properties.

3. Materials and Methods

3.1. Materials

All used materials/ chemicals were purchased from the following companies:

Polysciences, Inc., Warrington, USA:

- Polyacrylic acid (PAA) of 30 kDa

Sigma-Aldrich, Steinheim, Germany:

- Polyacrylic acid (PAA) of 1.2, 8 and 15 kDa
- N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC)
- N-hydroxysuccinimide (NHS)
- 2-(N-morpholino)ethanesulfonic acid (MES)
- Valganciclovir hydrochloride (ValG*HCl)
- Ganciclovir (Gan)
- Sodium hydroxide (NaOH)
- Hydrochloric acid (HCl)
- Phosphate buffered saline pH 7.4 (PBS)
- Sodium azide (NaN₃)
- Acetonitrile (ACN)
- Methanol (MeOH)
- Trifluoroacetic acid (TFA)
- Triethylamine (TEA)
- Phosphoric acid 85% (H₃PO₄)
- Monobasic sodium phosphate (Na₂HPO₄)
- Dibasic sodium phosphate (NaH₂PO₄)
- Sodium chloride (NaCl)
- Acetic acid (CH₃COOH)
- all other chemicals

3.2. Synthesis of PAA-ValG

Chemicals:

- Polyacrylic acid xx KDa (in H₂O)
- N-hydroxysuccinimide (NHS)
- 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)
- Valganciclovir hydrochloride
- MES-buffer: c(2-(N-morpholino)ethanesulfonic acid) = 1 mol/l , adjusted to pH 5.5 with NaOH
- c(HCl) = 6 mol/l
- c(NaOH) = 6 mol/l

Step 1. Activation of –COOH groups:

The polyacrylic acid solution was dissolved in MES-buffer, NHS dissolved in MES-buffer and EDC dissolved in MES-buffer were added. The pH value was adjusted with HCl or NaOH solution to pH 5. The pH value was monitored over the reaction time of 30 min at room temperature and adjusted when necessary.

Step 2. Conjugation:

Valganciclovir*HCl was dissolved in 0.001 M HCl and added to the reaction mixture from Step 1. The pH value was adjusted with HCl or NaOH solution to pH 5.75 – 6.00. The pH value was checked over the reaction time of 1 h at room temperature and adjusted when necessary.

Step 3. Purification:

The reaction mixture was adjusted to pH 3.0 with 6 M HCl. The white crystalline powder was washed with 0.001 M HCl, resuspended in 0.001 M HCl and lyophilized.

3.3. Characterization of the valganciclovir conjugated polyacrylic acid

The characterization included NMR spectroscopy, the quantification of impurities, the degree of conjugation and the amount of free drug in the substance. The release of ganciclovir was studied as it is one of the most important properties of the conjugate.

3.3.1. Identification (NMR spectroscopy)

PAA-ValG samples and valganciclovir were dissolved in DMSO-d₆ and proton NMR spectra was recorded with a Bruker BBFO 400 MHz NMR spectrometer. The spectra were compared with NMR data from valganciclovir in DMSO-d₆ from literature [59].

3.3.2. Quantification of ganciclovir and related substances

A highly sensitive HPLC method was developed and validated for the quantification of ganciclovir, valganciclovir and related substances under the given conditions:

Chromatographic conditions:

Detector: UV 254 nm

Column: 4.6 mm × 25 cm; 5 µm; ZORBAX XDB-Phenyl

Column temperature: 30 °C

Flow rate: 1.7 ml/min

Injection volume: 25 µl

Eluent A: Add 2.5 mL of triethylamine to 1000 mL water and adjust with trifluoroacetic acid to pH 3.0 ± 0.05. Mix this solution with 75 mL methanol.

Eluent B: Add 2.5 mL of triethylamine to 400 mL water and adjust with trifluoroacetic acid to pH 3.0 ± 0.05. Mix this solution with 675 mL methanol.

Gradient table:

Table 3.1: Gradient table for assay and degradation/ purity:

Time (min)	Eluent A (%)	Eluent B (%)
0.0	100	0
6.0	100	0
19.0	0	100
19.5	100	0
25.0	100	0

Note: For release testing, eluent A isocratic for 8 min run time.

Example chromatogram:

Figure 3.1.: Chromatogram of valganciclovir*HCl standard solution (0.00125 mg/ml)

Component table:

Table 3.2: Components of valganciclovir standard solution

Peak	Retention Time (min)	Area (%)	Name
1	3.251	1.0	Ganciclovir
2 - 3	5.602 + 5.981	99.0	Valganciclovir 1+2

3.3.2.1. Determination of Assay

Sample preparation:

25 mg of the polymer sample was dissolved and hydrolyzed in 10 ml 1 M NaOH by stirring at room temperature until a constant concentration was achieved. 0.5 ml of this solution were mixed with 4.5 ml of 0.001 M HCl, followed by 5 ml of 0.15 M H₃PO₄. The solutions were centrifuged and the supernatant was transferred into HPLC vials.

Calculation*:

$$\text{Assay} = \% \text{ Gan (w/w)} = 100\% \frac{w_{\text{Gan in sample}}}{w_{\text{PAA-ValG in sample}}}$$

*The exact sample weight, as well as dilutions were considered.

3.3.2.2. Determination of Free Drug

Sample preparation:

10 mg of the polymer sample were suspended in 20 ml 0.001M HCl by stirring until constant concentrations at room temperature. Afterwards, the solutions were centrifuged and the supernatant was transferred into HPLC vials.

Calculation*:

$$\% \text{ Free Drug} = 100\% \frac{C_{\text{Free Drug in sample}}}{C_{\text{Gan in sample}}}$$

*The exact sample weight, as well as dilutions were considered.

3.3.2.3. Determination of Degradation products and Impurities

Sample preparation:

The sample solutions from Assay and Free Drug determination were analyzed for impurities and presence of degradation products.

Calculation*:

$$\% \text{ Impurity} = 100\% \frac{C_{\text{Impurity in sample}}}{C_{\text{Gan in sample}}}$$

*The exact sample weight, as well as dilutions were considered

3.3.2.4. *In vitro* release testing

Release Media (PBN pH 7.4):

2.5 g NaCl, 8.5 g Na₂HPO₄, 1.5 g NaH₂PO₄ and 0.5 g NaN₃ were dissolved in 1000 ml H₂O and the pH was adjusted to pH 7.4 (7.40-7.44) with NaOH.

Sample preparation:

25 mg of PAA-ValG conjugate were weighed into a 250 ml volumetric flask and filled up with release medium to the mark. The volumetric flask was shaken at 50 rpm in a 37 °C environment. At each pre-determined time point, 500 µl of the clear supernatant was removed and diluted with 250 µl of 0.15 M H₃PO₄ into HPLC vials. The samples were stored in the refrigerator (2-8 °C) until they were analysed with HPLC.

Calculation*:

$$\% \text{ Released} = 100\% \frac{C_{Gan \text{ released}}}{C_{Gan \text{ in sample}}}$$

*The exact sample weight, as well as dilutions were considered.

3.3.3. Quantification of ganciclovir in vitreous humor

The quantification of ganciclovir in vitreous humor containing release samples required a more selective HPLC method than *in vitro* release testing, due to a more complex sample matrix:

Chromatographic conditions:

Detector: UV 254 nm

Column: 4.6 mm × 15 cm; 5 µm; ZORBAX 300-SCX

Column temperature: 30 °C

Flow rate: 2 ml/min

Injection volume: 5 µl

Eluent:

0.05 % (v/v) trifluoroacetic acid in a mixture of acetonitrile and water 50:50 (v/v)

Example Chromatogram:

Figure 3.2.: Chromatogram: ganciclovir in vitreous humor (green), blank vitreous humor (blue)

3.3.3.1. *Ex vivo* release testing (solid state)

Release testing of PAA-ValG samples (1 mg/ml) was performed in PBS pH 7.4, as well as in vitreous humor + 0.05% (w/v) sodium azide at 37 °C for 9 days. Samples were taken after 2 and 9 days to estimate the effect of the in vitreous humor containing esterase and other ingredients, on the drug release.

3.3.3.2. *Ex vivo* release testing (soluble state)

Release testing was performed in vitreous humor obtained from pigs, mixed with PBN pH 7.4 to ensure that the pH value stayed constant during the entire release testing of up to 7 days. Since it was not possible to perform a whole release experiment in vitreous humor, pre-released PAA-ValG samples had to be used. For this purpose, *in vitro* release samples of PAA-ValG in PBN pH 7.4 (0.1 mg PAA-ValG / ml) which were incubated at 37 °C for 6 months were used. These pre-released samples were mixed 1:1 with vitreous humor and incubated at 37° C. The same was done with a blank sample, consisting of PBN (pH 7.4) and vitreous humor, as well as the pre-released samples in PBN pH 7.4. After 7 days, the sample and reference solutions were mixed with PBN pH 7.4 or blank solution to a total ratio of sample or reference / PBN pH 7.4 / vitreous humor (1 / 1 / 1). Then 0.15 M H₃PO₄ in acetonitrile was added to quench further release by bringing the pH to ~3 and to precipitate most of the containing proteins. The samples were centrifuged, and the supernatant was transferred into HPLC-vials for analysis.

3.4. Methods for mechanistic studies

3.4.1. Synthesis and of Ac-ValG

Ac-ValG was synthesized by EDC coupling of acetic acid and valganciclovir under similar conditions as how the PAA-ValG conjugates were produced (see 3.2 Synthesis of PAA-ValG), with the exception that the synthesis was performed in acetate buffer to increase the stoichiometric ratio of acetic acid.

3.4.2. Quantification of Ac-ValG

Ac-ValG was quantified, using the method as used for the quantification of ganciclovir and related substance. (see 3.3.2 Quantification of ganciclovir and related substances)

3.4.3. Identification of Ac-ValG

The identification was performed, by mass spectrometry using an Agilent 1290 LC-MS system.

3.4.4. Reaction constant of Ac-ValG and valganciclovir

The Ac-ValG and valganciclovir containing reaction mixture was diluted with PBN stock solution, the pH was adjusted with diluted NaOH or HCl solution to the desired value and the volumes were made up with water to achieve a concentration of 0.1 mg/ml Ac-ValG and 0.02 mg/ml valganciclovir in PBN. The samples were placed in a 37 °C oven (with one sample in 60 °C).

At each time point, 500 µl of the sample were mixed with 250 µl of PBN pH 7.4 and 250 µl of 0.15 M H₃PO₄ in HPLC vials and stored at 2-8 °C until the HPLC analysis.

3.4.5. Glass transition temperature (calorimetric analysis)

The calorimetric response of PAA-ValG was measured with a DSC Q10 (TA Instruments, USA). The samples were placed in aluminum pans with empty aluminum pans as reference. After equilibrating to 40 °C, the samples were cooled at a rate of 1 °C/min to -70 °C, followed by heating at 1 °C/min to 40 °C. This cycle was repeated 3 or 4 times. The energy release on heating was visualized by plotting the derivative heat flow and used to determine the T_g.

3.4.6. Degree of hydration by water content gravimetrically

Around 65 mg PAA-ValG were compressed to tablets by using a FTIR pellet pressing device of 13 mm diameter. The compression force per tablet was 100 kN (0.75 kN / mm²). The tablets were swollen in 20 ml PBS pH 7.4 which was changed regularly to ensure that the pH stayed constant. At the desired time points, tablet pieces were removed, and the water content was measured as the loss of drying (LOD) using a TA Instruments TGA2950:

Figure 3.3.: Example TGA (green) and DTG (blue) curve of PAA-ValG 15-2 tablet piece after 6 weeks in PBS

The heating rate for the TGA was chosen as 10 °C/min from RT until 80 °C and as 7.5 °C/min from 80 °C to 180 °C. The weight loss at 140 °C was used for the calculation of the water content. The difference between the weight loss at 140 °C and 160 °C was used to confirm that the LOD was constant.

The measured LOD was converted into the tablet swelling as % of the remaining tablet weight at the time point (**tx**) by the following formula:

$$\% \text{ swelling} = 100\% \frac{100\%}{100\% - \% LOD_{tx}}$$

3.4.7. Degree of hydration by tablet swelling gravimetrically

Around 220 mg PAA-ValG were compressed to tablets by using a FTIR pellet pressing device of 13 mm diameter. The compression force per tablet was 100 kN (0.75 kN / mm²). The tablets were swollen in 100 ml PBN pH 7.4. The pH level was checked regularly and adjusted where necessary.

At the sampling point, the tablets were removed from the solution, with surface water removed and weighted. The swelling was calculated as % of the initial tablet weight by the following formula:

$$\% \text{ swelling} = 100\% \frac{\text{Tablet weight at } T_x}{\text{Tablet weight at } T_0}$$

3.4.8. pH dependence of [H₂O] by tablet swelling gravimetric

Around 220 mg PAA-ValG were compressed to tablets by using a FTIR pellet pressing device of 13 mm diameter. The compression force per tablet was 100 kN (0.75 kN / mm²). The tablets were swollen in 100 ml PBN pH 7.4. The pH was checked regularly and adjusted where necessary.

At the sampling point, the tablets were removed from the solution, with surface water removed and weighted. The swelling was calculated as % of the initial tablet weight by the following formula:

$$\% \text{ swelling} = 100\% \frac{\text{Tablet weight at } T_x}{\text{Tablet weight at } T_0}$$

3.4.9. Titration of PAA-ValG

Stock solution:

50 mg PAA-ValG were dissolved in 10 ml 0.05 M NaOH solution.

Sample solution (before release):

4 ml freshly prepared (< 10 min old) stock solution + 5.75 ml H₂O + 0.25 ml 5 M NaCl

➔ equal to 20 mg/ml PAA-ValG in 0.02 M NaOH, containing 0.125 M NaCl.

Sample solution (after release):

4 ml released stock solution (1 h at 60 °C) + 5.75 ml H₂O + 0.25 ml 5 M NaCl

➔ equal to 20 mg/ml PAA-ValG in 0.02 M NaOH, containing 0.125 M NaCl.

Solutions were titrated with 0.01 M HCl, containing 0.125 M NaCl.

4. Synthesis and characterization of PAA-ValG

4.1. Introduction

Being able to synthesize a newly designed material is crucial for the development of every new product. This is especially important for pharmaceutical applications as the product should not contain possibly harmful impurities (e.g. reactants, side products or residual solvents) and the synthesis should be reproducible. Furthermore, the reaction product should be isolated in a sufficient stable/ storable form. The design of the synthesis and work-up protocol are the most important, so as to achieve the previously mentioned requirements and have to be confirmed by suitable characterization techniques, which will be detailed in this chapter.

4.2. Synthesis of PAA-ValG conjugate

The synthesis of PAA-ValG conjugate was conducted from the starting materials valganciclovir hydrochloride and polyacrylic acid by coupling:

Figure 4.1.: Synthesis of PAA-ValG conjugate

Valganciclovir consists of ganciclovir and L-valine as spacer for the polymer drug conjugate. The fact that valganciclovir is an approved prodrug reduces the risk of unwanted side effects due to non-reacted excipients in the polymer drug conjugate, as these could appear when using other spacers (e.g. amino acids) which have not been tested as ganciclovir esters. Since valganciclovir is available in pharmaceutical quality at a reasonable price, no multiple-step organic synthesis for forming an ester of ganciclovir had to be performed.

Polyacrylic acid was chosen as polymer backbone for the conjugate, because it has more carboxylic acid groups per gram of polymer than most of the commonly used negatively charged polymers. This is important to prepare polymer drug conjugates with high drug loading, since only limited quantities/ volumes of formulations can be applied to the eye. Also, advantages of its biocompatibility, mucoadhesive properties and that it is already used in pharmaceutical formulations make it an excellent material choice.

The coupling was performed in aqueous buffer solution with 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS). These substances were chosen to keep all excipients and reaction products except the PAA-ValG conjugate water soluble, which eliminated the need of organic solvents during reaction and purification of the PAA-ValG conjugate.

Furthermore, these substances have also been used for coupling in biological applications and were expected to be easier to remove and are less harmful than coupling reagents which are commonly used.

The pH during conjugation was kept at pH 6 as a compromise between the high stability of valganciclovir at acidic pH and high reaction speed of carbodiimide couplings at higher pH values.

The reaction followed the shown mechanism, where R1 is polyacrylic acid, R4 is valganciclovir, substance (2) is EDC and substance (7) is the PAA-Val conjugate:

Figure 4.2.: Mechanism of EDC/ NHS coupling [60]

4.3. Molecular weight and stoichiometric ratios

The synthesis was performed with PAA of different molecular weight and in the following stoichiometric ratios:

Table 4.1: Stoichiometric ratios for the synthesis of PAA-ValG:

Name	MW of PAA (kDa)	Stoichiometric ratios			
		PAA R-COOH	NHS	EDC	ValG
PAA-ValG 1.2-2	1.2	1	2	2	1.1
PAA-ValG 8-2	8	1	2	2	1.1
PAA-ValG 15-2	15	1	2	2	1.1
PAA-ValG 30-2	30	1	2	2	1.1
PAA-ValG 8-1.5	8	1	1.5	1.5	1.1
PAA-ValG 15-1.5	15	1	1.5	1.5	1.1
PAA-ValG 30-1.5	30	1	1.5	1.5	1.1
PAA-ValG 8-1	8	1	1	1	1.1
PAA-ValG 15-1	15	1	1	1	1.1
PAA-ValG 30-1	30	1	1	1	1.1
PAA-ValG 8-0.5	8	1	0.5	0.5	0.55
PAA-ValG 15-0.5	15	1	0.5	0.5	0.55
PAA-ValG 30-0.5	30	1	0.5	0.5	0.55

These variations were intended to produce conjugates of different molecular weight and drug loading, so their effects can be studied.

4.4. Identification (NMR spectroscopy)

The confirmation of the proposed chemical structure is highly important for the development of new drug delivery systems.

For this purpose, PAA-ValG samples and valganciclovir were dissolved in DMSO-d₆ and proton NMR spectra was recorded with a Bruker BBFO 400 MHz NMR spectrometer. The spectra were compared with NMR data from valganciclovir in DMSO-d₆ from literature [59].

The NMR spectra of PAA-ValG showed that the protons in of the NH₂ group from the L-valine were not detectable after conjugation, whereas the protons from the NH₂ group from the guanine were still detectable (Figure 4.3). This led to the conclusion that the coupling of valganciclovir to the polyacrylic acid resulted in the desired reaction product. A conjugation of the NH₂ group from guanine would lead to a much more stable amide bond between ganciclovir and the polymer backbone, which would result in an almost permanent bond under physiological conditions, which cannot be released as easily as the ester bond between ganciclovir and the L-valine linker.

Figure 4.3.: NMR spectra of PAA-ValG 15-2 (red) and valganciclovir (blue) in DMSO-d₆

This shows that the chosen reaction conditions led to the desired reaction product and can be applied to all synthesized PAA-ValG conjugates.

4.5. Drug content (assay)

Assay is one of the most important characteristics of a polymer drug conjugate, since it gives the amount of active ingredient which the conjugate contains.

The assay of ganciclovir in the PAA-ValG conjugate was determined, based on the hydrolysis in aqueous NaOH solution to constant concentration of ganciclovir:

Figure 4.4.: Assay of PAA-ValG as % ganciclovir (w/w)

It was observed that the stoichiometric ratio during reaction only effected assay of the conjugates with the lowest stoichiometric ratio (0.5), but was constant afterwards. This is expected to be caused by steric hindrance, where the remaining carboxylic acid groups cannot be accessed, no matter how much coupling reagent was added. As expected, the molecular weight of PAA had no effect on the assay when observed.

The drug loading for the higher stoichiometric ratios (> 0.5) was much higher than all other studies reported in literature [6-12]. This is a very promising sign for the development of a new release platform, since ganciclovir is administered in relatively high concentrations which necessitates a high drug loading.

Furthermore, the loading was very consistent over the 10 batches with an average of 42.0% $\pm$ 1.1% ganciclovir (w/w), demonstrating a highly reproducible method of synthesis.

4.6. Free Drug

The determination of non-conjugated drug (free drug) in a polymer drug conjugate is relevant; despite there being no harm in the use of free drug, it will lead to a burst release.

The amount of free drug in the PAA-ValG conjugates was determined, based on the extraction with 0.001 M aqueous HCl until constant concentration:

Figure 4.5.: Free Drug in PAA-ValG of higher stoichiometric ratios (> 0.5)

The amount of free drug in PAA-ValG samples of higher stoichiometric ratios was < 0.5% for all samples. The free drug was mainly unreacted valganciclovir from the synthesis, while the free ganciclovir was less than 0.1%. This showed that the washing of the solid PAA-ValG with 0.001 M HCl after synthesis was successful.

Table 4.2: Free Drug in PAA-ValG samples of lower stoichiometric ratios:

Name	Free valganciclovir (%)	Free ganciclovir (%)	Conjugated drug (mol %)
PAA-ValG 8-0.5	79.7	0.4	0.7
PAA-ValG 15-0.5	45.3	0.4	2.7
PAA-ValG 30-0.5	76.2	1	1.1

The samples of lower stoichiometric ratios showed that they contained significant quantities of free drug after dialysis of the soluble reaction product with 0.001 M HCl. These samples were not further studied, since 2.7% or less of the COOH groups were conjugated.

4.7. Determination of degradation products and impurities

Individual impurities/ degradation products were measured in the PAA-ValG solutions from Assay which would contain possibly conjugated impurities, as well as in the solution for the determination of free drug in 0.001M HCl, which would contain soluble or unconjugated impurities.

There were two impurities / degradation products detectable:

Figure 4.6.: Chromatogram of PAA-ValG 15-2 after hydrolysis in NaOH

Peak 1 was in all studied samples measurable with 0.1% or less and was assigned to be guanine, a degradation product of valganciclovir. This was confirmed by retention time, which also excluded the L-valine, which was used as linker or solvent impurities (blank solution).

Peak 3 was only in NaOH hydrolyzed chromatograms detected and is constant from the first time of sampling in all samples and did not increase with longer hydrolysis time. It was not further analyzed, since the quantity is < 0.2%.

From these results, we concluded that our synthesis method gave high product quality as no impurities or degradation products of more than 0.1% were found in all the PAA-ValG conjugates.

4.8. Storage stability of PAA-ValG

The storage stability of PAA-ValG plays an important role, since there might be some time between the synthesis and application/characterization of the product, in which no drug should be released.

To determine the storage stability, one sample per molecular weight was analyzed for Assay, Free Drug and degradation products directly after synthesis/ lyophilization and again after 4 months of storage in a closed centrifuge tube at room temperature, with the following results observed:

Table 4.3: Storage stability of PAA-ValG powder at room temperature:

Name	Directly after synthesis			4 months after synthesis			
	Assay as % Gan (w/w)	FD % ValG	FD % Gan	Assay as % Gan (w/w)	FD % ValG	FD % Gan	% Assay after storage
PAA-ValG 1.2-2	42.7	0.1	< 0.1	40.3	0.1	< 0.1	94.4
PAA-ValG 8-2	42.0	0.4	< 0.1	40.0	0.4	0.1	95.3
PAA-ValG 15-2	40.5	0.2	< 0.1	39.1	0.2	0.1	96.4
PAA-ValG 30-2	42.5	0.1	< 0.1	40.8	0.1	0.1	96.0

There was no relevant increase in the free drug, which showed that PAA-ValG was not releasing any drug during storage in the solid state, even when there was water present. This was expected, since the purification was performed with 0.001 M HCl (pH 3), where ganciclovir esters is known to be very stable [61].

It was also observed that the Assay of all 4 samples decreased by 4.5% on average when compared to the initial value, but there was no increase in degradation products (all < 0.2%) detected. This led to the conclusion that the decrease in Assay was due to water uptake from the environment, which although not harmful, but should still be considered by weighing the substance for quantitative measurements or formulations shortly after the Assay determination.

4.9. Conclusion

The synthesis of PAA-ValG of different molecular weight was successful, when using coupling reagents in the ratio of 1, 1.5 or 2 molecules of EDC and NHS per carboxylic acid group of PAA.

Selective coupling of valganciclovir occurred using the NH_2 group from L-valine, instead of the unwanted coupling from the NH_2 group of ganciclovir was confirmed via NMR spectroscopy.

The drug loading was determined via hydrolysis of PAA-ValG in aqueous NaOH solution, followed by quantification of the released ganciclovir via HPLC. The Assay was found to consist of 42.0% ganciclovir (w/w) and the result was very consistent over the 10 batches produced, which had only 1.1% standard deviation. An effect of molecular weight or stoichiometric ratio on the assay was not observed, which means that the synthesis is highly reproducible, even if the quality of the raw materials might vary.

Free drug was determined by extraction of unconjugated valganciclovir and ganciclovir from PAA-ValG with 0.001M HCl. The percentage of free drug was less than 0.5% for all samples, so this means that there should be no significant burst release from free drug expected.

The determination of impurities and degradation products in the HPLC samples from assay and free drug showed that there were no possibly harmful impurities or degradation products ($> 0.1\%$) present, which spoke for the high quality of the chosen synthesis and purification process.

A measurement of the above-mentioned parameters after multiple months of storage demonstrated that PAA-ValG does not release any drug or degrade in relevant quantities, but that it took up humidity from the environment. This requires the accurate weighing of PAA-ValG for formulating, or quantitative measurements to be performed a short time after the assay determination.

5. Application of PAA-ValG for the treatment of CMV

5.1. Introduction

This chapter focuses on determining the suitability of PAA-ValG as a good candidate for *in vivo* studies, with the goal to control cytomegalovirus retinitis with fewer intravitreal injections as in the current treatment, which was described in Section 2.2. For this purpose, the release properties *in vitro* and *ex vivo* were determined and discussed, based on the required dosage. Toxicity, pharmacological activity and clearance of PAA-ValG were discussed, based on similar systems reported in literature.

5.2. Release testing

5.2.1. *In vitro* release testing

The release properties of every sustained release drug formulation are beside the assay, degradation and impurity determination most relevant. The *in vitro* release behavior helps to predict the *in vivo* release behavior, which was important to elucidate how much drug substance was released at every time point during application.

Release testing of all PAA-ValG samples in PBN pH 7.4 (n = 2) was performed:

Figure 5.1.: Release of ganciclovir from PAA-ValG.

All samples showed very similar release profiles, except for the PAA-ValG 1.2-2 sample which had a faster release at the beginning. It also seems that the other samples showed a molecular weight dependent release, but cannot be confirmed with the data plotted.

To evaluate the effects of different molecular weights, all release profiles per molecular weight were averaged:

Figure 5.2.: Release of ganciclovir from PAA-ValG, averaged by MW.

A molecular weight dependent release was confirmed, showing a faster release at lower molecular weight, however, the standard deviation for each time point was below 2.5% in the molecular weight range which was relevant for the application (PAA of 8 to 30 kDa).

Even though the different molecular weights of PAA-ValG showed similar release profiles, the *in vivo* release behavior was expected to be different, due to molecular weight dependent clearance from vitreous humor, as discussed in Section 5.3.3.

To evaluate the effects of the different stoichiometric ratios during conjugation, all release profiles per stoichiometric ratio (without 1.2-2) were averaged and plotted:

Figure 5.3.: Release of ganciclovir from PAA-ValG, averaged by stoichiometric ratio.

Stoichiometric ratios were observed to not have any significant effect on the release profiles during conjugation. The standard deviation is below 1.0% for all time points. This led to the conclusion that all produced conjugates of the same MW were similar. In further experiments, the used conjugates were reported, but seen as representative for all samples of the same molecular weight.

When using the PAA-ValG conjugates for the treatment of CMV retinitis, the release rate of ganciclovir is the most important factor since it gives us the amount of ganciclovir released during each time point of the application. The following release rates were observed and the average (excluding PAA-ValG 1.2-2) indicated above:

Figure 5.4.: Release rate of ganciclovir from PAA-ValG.

It was observed that the average release rate (as indicated in Figure 5.4) was decreasing over time, from 8.2% per month in the 1st month, to 2.2% per month in the 12th month, which was relatively constant and longer, when compared to other reported ganciclovir releasing systems [6-12].

With the observed release rate, 2 to 3 injections of around 11 mg PAA-ValG (depending on the assay), equivalent to 4.5 mg ganciclovir would have to be performed, to obtain the required dosage of 4.5 mg sustained ganciclovir release over 6-8 months [14].

As such, this would be an enormous improvement in the treatment of CMV, which required more than 25 injections within 6 month with the current treatment [45, 46].

Further *ex vivo* and *in vivo* experiments are needed to determine the exact dosage, however, it is a definite improvement of the current treatment as shown by *in vitro* release testing.

5.2.2. *Ex vivo* release testing

The use of aqueous buffers for *in vitro* release testing is well established, but the observed release profiles *in vivo* might be significantly different due to different ingredients of the biological fluids e.g. esterase. Cavallaro et. al. reported a 7 to 8 times faster release of ganciclovir from a polymer drug conjugate in human plasma when compared to a buffer of pH 7.4 [12]. On the other hand, D'Souza et. al. reported that enzymes cannot enter polymer drug conjugates in the solid state (e.g. hydrogels, micelles) [13].

Based on the two possible states of PAA-ValG in vitreous humor (VH), *ex vivo* release testing was performed in both the solid and soluble state of PAA-ValG.

5.2.2.1. *Ex vivo* release testing (solid state)

Release testing of PAA-ValG samples (1 mg/ml) was performed in PBS pH 7.4, as well as in vitreous humor (+ 0.05% (w/v) sodium azide) at 37 °C for 9 days. Samples were taken after 2 and 9 days to estimate the effect of the sample in VH-contained esterase and other ingredients on release:

Table 5.1: Release of ganciclovir from PAA-ValG conjugate in vitreous humor and PBS:

Name	Release time days	Release in PBS pH 7.4 (% per day)	Release in pig vitreous (% per day)	Release in VH / Release in PBS
PAA-ValG 15-2	2	0.021	0.029	1.3
PAA-ValG 15-2	2	0.024	0.022	1.0
Average	2	0.023	0.026	1.1
PAA-ValG 15-2	9	0.013	0.021	1.5
PAA-ValG 15-2	9	0.015	0.017	1.2
Average	9	0.014	0.019	1.4

Over 9 days of release at 37°C, an approximately 1.4 times faster release rate in vitreous humor (VH) was observed, as compared to that in PBS pH 7.4. Even though there was a shift of pH to 8.0 for VH and to 7.2 for PBS, which would have promoted a faster release, the difference in release rate was not significant. This experiment confirmed that PAA-ValG cannot be accessed by enzymes in the solid state, as it was reported for polymer drug conjugates by D'Souza et. al. [13], so that the initial release profile *in vivo* should be similar to the observed *in vitro* release profiles from Section 5.2.1.

5.2.2.2. *Ex vivo* release testing (soluble state)

Release testing or pre-released PAA-ValG samples was performed in PBN pH 7.4, as well as in a mixture of PBN pH 7.4 vitreous humor (1:1) for one week to estimate the effect of the VH-containing esterase and other ingredients during release:

Table 5.2: Release of ganciclovir from PAA-ValG conjugate in vitreous humor and PBN:

Name	% drug released after 6 months	Release in PBN pH 7.4 (% per day)	Release in pig vitreous (% per day)	Release in VH / Release in PBN
PAA-ValG 1.2-2	47	0.21	0.68	3
PAA-ValG 1.2-2	46	0.38	1.47	4
PAA-ValG 8-1	38	0.26	2.25	9
PAA-ValG 8-1	37	0.25	2.59	10
PAA-ValG 15-1	34	0.33	1.90	6
PAA-ValG 15-1	34	0.26	2.51	10
PAA-ValG 30-1	32	0.25	2.61	10
PAA-ValG 30-1	32	0.10	2.60	27
Average	37	0.25	2.08	8

Over 7 days of release at 37°C, an about 8 times faster drug release rate in vitreous humor containing PBN pH 7.4 was observed, compared to only PBN pH 7.4.

This experiment confirmed that PAA-ValG showed a faster release due to enzymes present in the soluble state, as it was reported for soluble polymer drug conjugates by Cavallaro et. al. [12].

Since the release rate *in vitro* was observed to be decreasing over time, as shown in Figure 5.4, the enzymatic release can help to keep the release rate constant over time, and to utilize all of the injected drug.

In the best case, one injection of PAA-ValG was equivalent to one discontinued Vitrasert implant, where 4.5 mg of ganciclovir lasted 6-8 months [14]. This would require the enzymatic release to increase the release rate by 1.5 to 4.2 times over time, as shown in Table 5.3:

Table 5.3: Release rate of ganciclovir from PAA-ValG *in vitro* vs ideal release

Release rate (%/month)	Time in months							
	1	2	3	4	5	6	7	8
<i>In vitro</i> release	8.2	6.6	6.3	5.7	4.3	4.0	3.6	3.2
Ideal release 6 months	16.6	16.6	16.6	16.6	16.6	16.6	---	---
Times faster <i>in vivo</i>	2.0	2.5	2.6	2.9	3.9	4.2	---	---
Ideal release 8 months	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Times faster <i>in vivo</i>	1.5	1.9	2.0	2.2	2.9	3.1	3.5	3.9

5.3. Predicted *in vivo* properties of PAA-ValG

5.3.1. Toxicity

The toxicity of polymers is related to their chemical structure and is mainly caused by the interaction between positively charged polymers and negatively charged biological membranes. Polymers with positive charge are often toxic, whereas polymers with neutral or negative charge are not, when applied intravitreally or on the ocular surface [62] [50]. Existing information of the following structural related polymers were used to predict the toxicity of PAA-ValG:

Table 5.4: Chemical related polymers with known toxicity:

Substance	Structure	Comment
Polyacrylic acid (PAA)		PAA is used in many pharmaceutical formulations (e.g. ganciclovir ophthalmic gel 0.15%) [3]. PAA and PAA copolymers are considered pharmaceutically safe for ophthalmic applications [63] [64].
Poly-N-isopropylacrylamide (PNIPAM)		PNIPAM is a temperature-responsive polymer which can be injected at room temperature and gels when heating up to body temperature. It has been intensively studied for drug delivery[64]. Intravitreal injections of 0.1 mL 50% PNIPAM were nontoxic in rabbits (n=12) [65]
Poly-N-(2-hydroxypropyl) methacrylamide (PHPMA)		HPMA copolymers are frequently used as carrier for low molecular weight drugs (mainly for cancer treatment) to enhance therapeutic efficacy and limit side effects. They were initially developed as plasma expanders, are highly hydrophilic, non-immunogenic and non-toxic [64]

As such, PAA-ValG is expected to be pharmaceutically safe, non-immunogenic and non-toxic.

5.3.2. Pharmacological activity

The pharmacological activity of PAA-ValG is relevant, since a quantity with a ganciclovir content of multiple conventional injections will be injected at once.

For polymer drug conjugates with the chemotherapy drug *doxorubicin*, it is known that the conjugate is much less active (cytotoxic) than the free drug solution and the cytotoxic effect seen when incubating cells with the conjugate comes from a combination of conjugate and free drug toxicity [50].

Since ganciclovir is acting inside affected cells, it is expected that PAA-ValG will not show pharmacological activity due to the following reasons:

- PAA-ValG cannot easily penetrate into the cells due to its negative charge and molecular weight which is much higher than that of free ganciclovir.
- The viral thymidine kinase which is phosphorylating ganciclovir to ganciclovir monophosphate will be sterically hindered due to the attached polymer.
- The cellular enzymes, which are phosphorylating ganciclovir monophosphate to ganciclovir diphosphate followed by ganciclovir triphosphate will be sterically hindered due to the polymer.

Figure 5.5.: Phosphorylation of ganciclovir

The pharmacological active ganciclovir triphosphate which inhibits the CMV DNA polymerase (UL54) and stops the replication of viral DNA can only be produced from free ganciclovir, but not from PAA-ValG-conjugated ganciclovir.

5.3.3. Elimination from the vitreous

The elimination of PAA-ValG from the vitreous after injection is expected to happen via physical elimination of the releasing/ biotransforming polymer drug conjugate into to the blood stream, crossing blood-ocular barriers. These consist of anterior blood-aqueous barrier (BAB) and posterior blood-retinal barrier (BRB) as shown by Raviola:

Figure 5.6.: Blood-ocular barriers: tight (—) and leaky (---) endothelium/epithelium [53]

The effect of the molecular weight (molecular radius) on the speed of elimination (permeability coefficient) was shown by Pitkänen et al. on the example of carboxyfluorescein and fluorescein isothiocyanate (FITC)-labeled dextrans with molecular masses from 4 to 80 kDa across RPE-choroid:

Figure 5.7.: Permeability of fluorescent dextrans as function of molecular radius [54]

PAA-ValG is expected to be eliminated according to the following scheme:

Figure 5.8.: Scheme for the elimination of PAA-ValG from Vitreous

It is assumed that PAA-ValG will be mainly in the solid state after intravitreal injection, due to limited solubility at physiological pH. In this hydrated, but solid state, ganciclovir is released into the vitreous fluid, but the solid PAA-ValG was not eliminated via blood-ocular barriers.

With increasing hydrophilicity due to the release of ganciclovir, more and more partially released PAA-ValG will get dissolved into the vitreous fluid, while the release of ganciclovir continues at a faster rate than in the solid state, since enzymes from vitreous can now enter the conjugate.

The soluble PAA-ValG can now be eliminated via blood-ocular barriers, where by the continuously decreasing molecular weight (molecular radius) of the polymer has a significant effect on the speed of elimination (permeability coefficient).

This property should cause the release inside the vitreous humor to continue until almost all drug is released.

5.4. Conclusion

The performed release testing experiments can be classified into *in vitro* release testing, *ex vivo* release testing in the solid state, as well as *ex vivo* release testing in the soluble state.

The *in vitro* release testing in a simulated ocular environment showed a sustained release over more than 12 months, with a decreasing release rate from 8.2% per month in the 1st month, to 2.2% per month in the 12th month.

The *ex vivo* release testing in the solid state, using vitreous humor from pigs showed no significant change in the release rate, compared to *in vitro* release conditions.

The *ex vivo* release testing in the soluble state, using a mixture of vitreous humor from pigs and *in vitro* release medium showed an 8 times faster release rate, compared to *in vitro* release conditions.

Considering that PAA-ValG was in a solid (gel/ hydrogel-like) state after injection and only dissolving during the release, based on the concentration and the molecular weight, it was expected that the observed *in vivo* release would be similar to the *in vitro* release at the beginning, but faster after PAA-ValG has dissolved. This would result in a release that is more constant, but faster, which might only require a single injection of PAA-ValG for a treatment duration of 6 to 8 months.

The properties of PAA-ValG after intravitreal injection (*in vivo*) were predicted, based on comparison with polymers of a similar chemical structure reported in literature. The following predictions were made:

1. PAA-ValG is pharmaceutically safe, non-immunogenic and non-toxic.
2. PAA-ValG is not pharmacological active.
3. PAA-ValG gets eliminated via blood-ocular barriers, whereby the continuously decreasing molecular weight of the conjugate has a significant effect on the speed of elimination.

Considering the high drug loading, purity, stability, long-term sustained release, and predicted properties from literature, this suggests that PAA-ValG would be an excellent candidate for the *in vivo* studies.

6. Mechanistical studies of in vitro drug release

6.1. Introduction

The release of ganciclovir from PAA-ValG conjugate is based on the hydrolysis of the ester bond between the L-valine linker (R) and ganciclovir (R'):

Figure 6.1.: Alkaline ester saponification [66]

Even though the reaction of the two reactants (PAA-ValG and OH^-) is of a second-order reaction, the rate equation for fully hydrated carboxylate esters can be reduced to a pseudo-first-order rate equation, if the pH (hydroxide ion concentration) is kept constant.

The first-order reaction kinetics described by the following equation:

$$-\frac{d[E]}{dt} = k [E]$$

- $d[E] / dt$ describes the decrease in the concentration of cleavable drug $[E]$ over time, which is equivalent to the increase of released drug over time (release rate). In a first-order rate equation, the reaction speed is only dependent on the concentration of cleavable drug $[E]$, multiplied with a reaction constant k . The reaction constant k is specific for chemical structure, the pH and the temperature.

Since the drug release from the ester-linked polymer drug conjugates is known to be determined by a number of inter-dependent processes [15], the reaction constant k for the fully hydrated ganciclovir ester, as well as the factors which are making the drug release behavior different from a fully hydrated ester, were studied in this chapter.

6.2. Determination of the reaction constant k

The reaction constant of a first-order reaction is defined as the negative slope, when plotting the logarithmic concentration against time:

Figure 6.2.: First order reaction constant [67]

Even though the reaction constant for the hydrolysis of valganciclovir to ganciclovir at 37 °C and different pH values were determined by Stefanidis and Brandl [61], the coupling of an acyl group to L-Valine led to an increase of the pKa from 2.29 [68] to 4.11 [69], which is known to change the reaction constant.

This made it necessary to use an acylated valganciclovir to determine the reaction constant, and since the inductive effect which is responsible for the pKa change can only affect 3 neighboring atoms, acetylated valganciclovir was chosen as the model substance:

Figure 6.3.: Chemical structure of acetylated valganciclovir (Ac-ValG)

6.2.1. Synthesis and characterization of Ac-ValG

Ac-ValG was synthesized by EDC coupling of Acetic acid and valganciclovir under similar conditions as the PAA-ValG conjugates were produced, with the exception that the synthesis was performed in acetate buffer to increase the stoichiometric ratio of acetic acid and to reduce the number of components in the reaction mixture. The HPLC analysis of the diluted reaction mixture was performed, as indicated:

Figure 6.4.: Chromatogram of Ac-ValG reaction mixture.

Table 6.1: Components of Ac-ValG reaction mixture.

Peak	Retention Time (min)	Area (%)	Name	Identity (mass)
1	2.427	---	Buffer	---
2	3.180	1.0	Ganciclovir	confirmed
3 - 4	5.479 + 5.838	13.4	Valganciclovir 1+2	confirmed
4 - 5	13.484 + 13.660	85.6	Ac-ValG 1+2	confirmed
6	16.885	---	NHS-Acetate	confirmed

The analysis showed that Ac-ValG was present with a purity of 85.6% (area), in addition to the presence of 13.6% unreacted valganciclovir and 1.0% ganciclovir. This purity was sufficient for the determination of the reaction constant, as the decrease in the concentration of Ac-ValG and not the increase in the concentration of ganciclovir was used to determine the reaction constant. Furthermore, the decrease in the concentration of valganciclovir can be used to validate the methodology used.

The identity of the substances were confirmed via mass spectrometry and the fact that Ac-ValG formed ganciclovir when hydrolyzed. This would not have been the case should the ganciclovir be acetylated instead of the the L-valine.

6.2.2. Reaction constant of Ac-ValG

A reaction mixture containing Ac-ValG was diluted with PBN stock solution, while the pH level was adjusted with diluted NaOH or HCl solution to the desired value, and the volumes were made up with water to achieve a concentration of 0.1 mg/ml Ac-ValG in PBN. The samples were incubated at 37 °C (with one sample at 60 °C), and following decrease in the concentration of Ac-ValG was observed:

Figure 6.5.: First-order plots for the hydrolysis of Ac-ValG at 37°C

The decrease in Ac-ValG concentration was pseudo-first-order.

Table 6.2: Hydrolysis of Ac-ValG in numbers:

Release media	pH	Temp. (°C)	Conc. Ac-ValG (mg/ml)	k (1/h)	$T_{1/2}$ (h)	R^2
PBN	5.0	37	0.1	0.00000514	134854	0.9444
PBN	6.0	37	0.1	0.0000530	13068	0.9780
PBN	7.0	37	0.1	0.000481	1440.5	0.9908
PBN	7.4	37	0.1	0.00129	535.6	0.9989
PBN	8.0	37	0.1	0.00457	151.6	0.9996
PBN	7.4	60	0.1	0.02976	23.3	0.9971

6.2.3. Reaction constant of valganciclovir

To confirm the correct determination of the reaction constant for the hydrolysis of Ac-ValG without effects from ingredients of the reaction mixture, the decrease in the concentration of valganciclovir in the reaction mixture was also measured, so that the rate constant can be compared to previous results from Stefanidis and Brandel [61].

The following decrease in the concentration of valganciclovir was observed:

Figure 6.6.: First-order plots for the hydrolysis of valganciclovir at 37°C

The decrease in valganciclovir concentration was pseudo-first-order.

Table 6.3: Hydrolysis of valganciclovir in numbers:

Release media	pH	Temp. (°C)	Conc. valganciclovir (mg/ml)	k (1/h)	$T_{1/2}$ (h)	R^2
PBN	4.0	37	0.02	0.000365	1899	0.9994
PBN	5.0	37	0.02	0.00118	587.4	0.9991
PBN	6.0	37	0.02	0.00864	80.2	0.9997
PBN	7.0	37	0.02	0.05516	12.6	0.9997
PBN	7.4	37	0.02	0.07709	9.0	0.9996
PBN	8.0	37	0.02	0.09132	7.6	0.9992

6.2.4. Reaction constant discussion

The pH-rate profiles for the hydrolysis of Ac-ValG at 37 °C and 60 °C as well as the pH-rate profiles of valganciclovir at 37 °C were plotted:

Figure 6.7.: pH-rate profiles for the hydrolysis of Ac-ValG and valganciclovir

The measured pH-rate-profile of valganciclovir was similar to the reported one from Stefanidis and Brandel [61], so that the chosen method for the determination of the reaction constant can be seen as representative.

It was also observed that the pH-rate profile of Ac-ValG was monophasic, whereas the release profile of valganciclovir was biphasic, as Stefanidis and Brandel [61] described with the following formula:

$$k_{\text{obs}} = \frac{K_w}{[\text{H}^+]} \left(\frac{k_1[\text{H}^+] + k_2 K_a}{[\text{H}^+] + K_a} \right)$$

K_w is the autoprotolysis constant of water at 37 °C = $10^{-13.6}$

K_a is the ionization constant of the amine moiety of the valine group = $1.7 \cdot 10^{-7}$

$k_1 = 7 \cdot 10^5 \text{ M}^{-1} \text{ h}^{-1}$

$k_2 = 1.3 \cdot 10^3 \text{ M}^{-1} \text{ h}^{-1}$

The formula was derived from the following reaction scheme:

Figure 6.8.: Reaction scheme for the hydrolysis of valganciclovir [61]

Since the amino group of valganciclovir was converted into an amide group when synthesizing Ac-ValG, it cannot be protonated anymore. The measured pH-rate profile of Ac-ValG can be described with the following formula:

$$k_{\text{obs}} = \frac{K_w}{[\text{H}^+]} k_1$$

K_w is the autoprotolysis constant of water at 37 °C = $10^{-13.6}$

$$k_1 = 2.0 \cdot 10^3 \text{ M}^{-1} \text{ h}^{-1}$$

Measured and calculated reaction constant showed a good recovery:

Table 6.4: Hydrolysis of Ac-ValG calculated vs. measured:

pH	k (1/h) calculated	k (1/h) measured	Recovery %
5.0	0.00000502	0.00000514	97.7
6.0	0.0000502	0.0000530	94.8
7.0	0.000502	0.000481	104.4
7.4	0.00126	0.00129	97.8
8.0	0.00502	0.00457	109.9
Average:			100.9

To evaluate the pH value, which was equivalent to an observed reaction constant k (1/month), the following curve-fitting was performed:

Figure 6.9.: pH vs reaction constant k .

The following relationship was found:

$$pH = 0.4343 \ln (k [OH^-]) + 7.4421$$

For the opposite case, the following formula applies:

$$k [OH^-] = 4E - 08 e^{(2.3025 pH)}$$

6.2.5. Reaction constant conclusion

To determine the reaction constant for the hydrolysis of the ester bound between the polymer backbone + linker (PAA-Val), and ganciclovir in the fully hydrated state, a model substance had to be used, since PAA-ValG does not follow a pseudo-first-order, which meant that there were multiple factors affecting the rate of hydrolysis.

Even though the starting material valganciclovir showed the relevant bond and its hydrolysis rate constants at different pH were studied by Stefanidis and Brandel [61], the inductive effects of the acylation of the NH_2 group from L-Valine is changing its pK_a value, which also affect the reaction constant. This is why acylated (acetylated) valganciclovir (Ac-ValG) had to be synthesized by EDC coupling, in a similar way as PAA-ValG is synthesized, but using acetic acid instead of PAA.

The obtained reaction mixture containing 85.6% Ac-ValG, 13.4% valganciclovir and 1% ganciclovir, had its structures confirmed by mass spectrometry and hydrolysis profile were then diluted to 0.1 mg/ml Ac-ValG and 0.02 mg/ml valganciclovir with PBN of different pH and incubated. The reaction constants were calculated from the decrease in Ac-ValG and valganciclovir concentration over time.

The reaction constant of valganciclovir showed a biphasic pH-rate-profile, confirming the previous reported data from Stefanidis and Brandel [61], in which the pH-rate-profile for the hydrolysis of Ac-ValG depicted a linear relationship between pH and logarithmic rate constant. This can be explained by the difference in the chemical structures, in which the L-valine of valganciclovir can be present in two states (protonated and non-protonated), showing different inductive effects, where the L-valine in Ac-ValG was acetylated (NH_2 group transformed into an amide) so that no protonation would be possible, hence the inductive effect was independent of the pH.

The reaction constant for the ester hydrolysis of Ac-ValG was found to be directly proportional to the concentration of hydroxide ions $[\text{OH}^-]$ in the range of pH 5 to 8. This meant that the reaction constant will be 10 times faster per 1 pH unit increase.

6.3. Determining the degree of hydration [H₂O]

The degree of hydration describes the fraction of ganciclovir esters groups exposed to the hydrolyzing agent (water or hydroxide ions). The degree of hydration plays an important role to help explain the mechanism of release. There are two main factors known to affect the degree of hydration, which are free volume and hydrophobicity. Since there is no direct way to measure the degree of hydration, Pitt and Shah measured the water uptake during release/ hydrolysis by tablet swelling to approximate the degree of hydration [15].

6.3.1. Glass transition temperature / free volume

The glass transition temperature (T_g) is a relevant characteristic of drug releasing polymeric systems (e.g. PLGA particles or polymer drug conjugates), since a lower T_g (higher free volume) was shown to increase the polymer degradation/ drug release, due to its effect on the free volume [70].

The following derivative heat flow of PAA-ValG samples were recorded, when running multiple circles from 40 °C to -70 °C at heating and cooling rates of 1 °C/min:

Figure 6.10.: Calorimetric response of PAA-ValG samples

The observed signals in the heating curves represented the glass transition temperatures of the PAA-ValG conjugate, which increased with the molecular weight, as is usually observed in polymers of similar chemical structure and different molecular weight (e.g. PLGA of different molecular weights).

6.3.2. Hydrophobicity

When it comes to the hydrophobicity of polymers, the ratio of repeating units, as well as their degree of ionization are important. Following repeating units can be found:

Figure 6.11.: Different repeating units which PAA-ValG consists of during release

The ratio of the repeating units can be determined from the Assay and showed that around 25% of all groups were PAA-ValG, which got converted into PAA-Val over the release. All of the repeating units can be negatively charged, depending on the pH:

Table 6.5: Degree of ionization of repeating units in PAA-ValG

pH	PAA	PAA-ValG	PAA-Val
	pKa = 5.1-6.2*	pKa = 9.4*	pKa = 4.11*
5.0	0.28	0.00	0.89
6.0	0.60	0.00	0.99
7.0	0.86	0.00	1.00
7.4	0.91	0.01	1.00
8.0	0.96	0.04	1.00

*The degree of ionization of the shown repeating units was determined for PAA via titration of the conjugated PAA (Figure 6.12) and calculated for the ganciclovir and PAA-valine containing groups, based on their pKa value.

6.3.2.1. Determination of the degree of ionization

The degree of ionization was determined via back-titration of a PAA-ValG solutions in NaOH with HCl, where the molar consumption of NaOH at each pH was equivalent to the negatively charged groups. The titration was performed with two solutions:

Before release:

A freshly prepared PAA-ValG solution in 0.02 M NaOH + 0.125 M NaCl, seen as 0% released (previous stability experiments with HPLC).

After release:

An aliquot of the same solution, after complete release of ganciclovir at 60 °C, seen as 100% released (constant pH and stability experiments with HPLC).

Following consumption of NaOH (mmol) per gram of PAA-ValG was measured:

Figure 6.12.: pH-dependent ionization of PAA-ValG 30-1.

Experiments with other than the shown PAA-ValG conjugate confirmed the representative character of this sample, due to the similar chemical composition.

The following values were extracted from the titration curves:

Table 6.6: Ionization of PAA-ValG (constant mass) in numbers:

pH	Ionization of PAA-ValG (mmol NaOH / g PAA-ValG)			
	0% released	100% released	100% release -0% release	100% release / 0% release
5.0	1.40	3.30	1.90	2.36
6.0	3.10	5.30	2.20	1.71
7.0	4.45	6.75	2.30	1.52
7.4	4.75	7.10	2.35	1.49
8.0	5.10	7.50	2.40	1.47

It was shown that the degree of ionization of PAA-ValG increased with the pH, as well as with the release of ganciclovir. Even though the difference of ionization before and after release was the highest at a high pH level of pH 8, the ratio between both release showed the higher change at a lower pH level of pH 5.

A linear relationship of the ionization between 0 and 100% release would result in the following release-dependent ionization:

Figure 6.13: Release-dependent ionization of PAA-ValG 30-1 (constant mass).

Considering the mass change of PAA-ValG due to the release of 42.0% (w/w) ganciclovir in average, the following ionization per actual mass can be observed:

Table 6.7: Ionization of PAA-ValG (actual mass) in numbers:

pH	Ionization of PAA-ValG (mmol NaOH / g PAA-ValG)			
	0% released	100% released	100% release -0% release	100% release / 0% release
5.0	1.40	5.69	4.29	4.06
6.0	3.10	9.14	6.04	2.95
7.0	4.45	11.64	7.19	2.62
7.4	4.75	12.24	7.49	2.58
8.0	5.10	12.93	7.83	2.54

The qualitative effects of pH and release on the ionization compared to the calculation with constant mass were similar, but the value for the ionization per actual mass increased significantly.

A mass correction on the linear relationship of the ionization between 0 and 100% release would result in the following release-dependent ionization:

Figure 6.14.: Release-dependent ionization of PAA-ValG 30-1 (actual mass).

6.3.3. Determination of [H₂O] by water content gravimetric

Around 65 mg PAA-ValG were compressed to tablets by using a pellet pressing device of 13 mm diameter. The compression force per tablet was 100 kN (0.75 kN / mm²). The tablets were left to swell in 20 ml PBS pH 7.4, causing them to break:

Figure 6.15.: PAA-ValG tablets after 3 weeks swelling in PBS pH 7.4

The PBS was changed regularly to ensure that the pH remained constant. At the sampling point, tablet pieces were removed and the water content measured as the loss of drying (LOD), which was then converted into % swelling of remaining weight:

Figure 6.16.: [H₂O] by water content gravimetrically in PBS pH 7.4

It was shown that the initial hydration could be completed in less than 2 weeks for all samples and this was followed by continuous swelling, depending on the molecular weight of the sample. The MW-dependent swelling can be explained by the lower T_g (higher free volume) of the lower molecular weight samples as shown in Figure 6.10.

6.3.4. Determination of [H₂O] by tablet swelling gravimetric

Tablets of around 220 mg PAA-ValG were left to swell in 100 ml PBN pH 7.4. At the sampling point, the tablets were removed from the solution, with surface water removed and subsequently weighed. Though there was bending noticed during the initial hydration, this did not affect the final tablets:

Figure 6.17: Initial hydration of PAA-ValG 15-1.5 tablets (up left and up right) as well as hydrated tablets after 1 week in PBN 7.4 (PAA-ValG 8-1.5 down left, 15-1.5 down middle, 30-1.5 down right)

The following swelling for PAA-ValG tablets in PBN pH 7.4 at 37 °C was observed:

Figure 6.18.: Tablet swelling experiments in PBS pH 7.4

The swelling of the tablets was molecular weight dependent and increasing over time, as already discussed in the initial [H₂O] experiment by water content.

It can be seen that the initial hydration in PBN pH 7.4 was completed in less than one week, and is faster than the initial hydration in PBS pH 7.4. This was caused by the higher buffering capacity of PBN (when compared to PBS from the initial experiment), which allowed for a faster pH adjustment of the PAA-ValG tablets which were initially acidic. The initial acidic pH level was caused by the work-up of PAA-ValG after synthesis at pH 3.

In comparison to the experiment from Section 6.3.3, the standard deviation seen in the current experiment was much lower and the measurements easier to perform, but this also meant additional cost due to a higher sample quantity required. Furthermore, the PAA-ValG 8-1.5 tablet started falling apart after one week, so this method can only be applied to polymers which were able to form stable tablets. Another point to note would be that the PAA-ValG 15-1.5 and PAA-ValG 30-1.5 tablets lasted more than 6 months without visible damage to the tablet's shape.

6.3.5. pH dependence of [H₂O] by tablet swelling gravimetric

Tablets of around 30 mg PAA-ValG 15-2 were hydrated in PBS pH 7.4 at 37 °C for 2 weeks. Afterwards the buffer was exchanged to pH-adjusted PBS at pH levels 7.0, 7.4 or 8.0, with the swelling left to continue at 37 °C. At the sampling points below, the tablets were removed from the solution, with surface water removed and tablets weighed.

The following swelling for PAA-ValG tablets at different pH was observed:

Figure 6.19.: Swelling (hydration) of PAA-ValG tablets at different pH

It was observed that the swelling was pH dependent, as expected, due to the pH-dependent degree of ionization, as described in Section 5.3.2.1.

6.3.6. Homogeneity of the degree of hydration

While the degree of hydration was dependent on the entire polymer (tablet), the release of ganciclovir on the other hand was mainly dependent on the hydration of the ester bond between the L-valine linker and ganciclovir. The homogeneity of the hydration was analyzed by plotting the buffer capacity of PAA-ValG before and after release (data from titration experiment, Figure 6.12) against the pH value:

Figure 6.20.: Buffer capacity of PAA-ValG 30-1.

Based on the fact that the buffer capacity was strongest around the pKa of buffering groups, and that the buffer capacity increased with the concentration of buffering group, the following interpretation was made:

- 1) The increase of buffering capacity at pH 4 was due to the formation of PAA-Val groups (pKa = 4.11), when ganciclovir was released from PAA-ValG.
- 2) The constant buffering capacity in the range of pH 5 to pH 7 was due to the buffering capacity of PAA groups (pKa 5.1 to 6.2). The constant buffering capacity showed that the number of available (hydrated) PAA groups did not change significantly during the release.
- 3) The buffering capacity at pH 9 to pH 10 was due to the buffering capacity of ganciclovir (pKa 9.4).

A change in the buffering capacity at pH 9 to pH 10 during release can only be explained with a change in the hydration of ganciclovir. The release does not affect the pKa of the substance, as the ester bond is more than 3 atoms away from the point of ionization, which excludes inductive effects. In the following structure, R represents PAA-Val in the conjugated state or H in the released state:

Figure 6.21.: Ionization of ganciclovir

The hydration of PAA-ValG is not homogeneous, as the buffering capacity of PAA was observed to be constant. Where the buffering capacity of ganciclovir had increased with the release of ganciclovir, this meant that there was more PAA than ganciclovir hydrated in the initial phase.

6.3.7. Conclusion of the degree of hydration

The influencing parameters on the degree of hydration such as the free volume and the hydrophobicity, as well as the water intake into the material and the homogeneity of the hydration were studied in this section.

The glass transition temperature (T_g) of PAA-ValG was studied via thermogravimetric analysis. It was observed that the T_g increased with the molecular weight, which meant that the free volume as well as the degree of hydration are expected to decrease with the molecular weight [70].

The effect of the structure on the hydrophobicity was discussed and the different repeating units which PAA-ValG consists of during release including their pH dependent charge were shown. As the most important parameter for the hydrophobicity, the degree of ionization was determined via titration before and after release.

It was shown that the degree of ionization of PAA-ValG increased with the pH, as well as with the release of ganciclovir. Even though the difference in ionization before and after release was the highest at a high pH (pH 8), the ratio between both releases showed a higher change at a lower pH (pH 5).

The degree of hydration was determined via swelling experiments of compressed PAA-ValG samples, by using either an increase in the water content of a piece of material (loss of drying), or by an increase in the weight of the entire tablet in order to calculate the amount of swelling.

The degree of hydration was shown to increase with the release of ganciclovir for all studied samples and conditions. Furthermore, this decreased with an increasing molecular weight as expected from the glass transition temperature experiment. A pH-dependent swelling, with higher degree of hydration at a higher pH was also shown.

Experiments to determine the homogeneity of hydration via titration showed that though the concentration of accessible PAA groups remained constant during release, the concentration of accessible ganciclovir had increased during release. This meant that the hydration of PAA-ValG was not homogenous.

6.4. pH shift in microclimate of PAA-ValG during release

During the release of ganciclovir from PAA-ValG, the pH of the release medium was observed to have dropped when there was insufficient buffering capacity. Even though the buffer capacity of the surrounding release media was sufficient to keep the pH constant, the microclimate pH was expected to fall, as was reported for biodegradable polymers like poly (lactic-co-glycolic acid) during degradation (ester hydrolysis) [16].

To determine the maximum possible drop in the microclimate pH within PAA-ValG during release, a PAA-ValG sample was dissolved in diluted NaOH solution and an aliquot was titrated with HCl until the desired pH values were achieved. The remaining PAA-ValG solution was fully released (confirmed by HPLC) by heating it to 60 °C for one hour. With the same volume of HCl solution, as previously used to titrate to pH values of interest was added, the pH of the unbuffered solution changed as follow:

Table 6.8: pH-change of PAA-ValG 30-1 during release (unbuffered):

pH before release	6.00	7.00	7.40	8.00
pH after release	4.90	5.58	5.74	5.92
Δ pH	1.10	1.42	1.66	2.08
$10^{\Delta \text{pH}}$	13	26	46	120

It was observed that there was a bigger change in the pH at higher pH value, which can be explained by the buffering capacity of the non-conjugated PAA groups which was the strongest in the range of pKa 5.1-6.2. Furthermore, the increasing number of PAA-Val groups (pKa 4.11) during release lost its strength with a decreasing pH (e.g. at pH 8.0, 100% of the COOH were dissociated, however at pH 4.11, only 50% of the protons dissociated).

Even though the pH difference of 2.08 (at pH 8.0) to 0.82 (at pH 5.0) may not be significant, from the logarithmic scale viewpoint, the reaction constant had changed by a factor of 10 per pH unit. Considering that, the maximum change in the reaction constant during release at the initial pH of 6.0 would be 13 times slower, but this would translate to be 120 times slower at pH 8.0.

To predict the microclimate pH between the determined values in the experiment without the need to hydrolyze samples to specific concentrations of cleavable drug [E], the titration curves at 0% release from Figure 6.12. were re-calculated with the knowledge that the release of ganciclovir did not significantly change the buffering capacity of PAA-groups in the relevant pH range, as shown in Figure 6.20. The quantity of hydroxide ions which was consumed upon the release of ganciclovir was subtracted from the added NaOH and the resulting pH plotted against the concentration of cleavable drug [E] left:

Figure 6.22.: pH shift in microclimate of PAA-ValG during release

From Figure 6.20, it can be inferred that the increase of buffering capacity with decreasing pH had caused a shaper drop in the pH at the beginning of the release. In a buffered environment, the drop in the microclimate pH within PAA-ValG was expected to be lower, but this might still be present.

6.5. Release rate analysis and discussion

To explain the observed release behavior (release rates), previously measured parameters such as the reaction constant k , the degree of hydration $[\text{H}_2\text{O}]$, the pH shift in the microclimate $[\text{HO}^-]$, the concentration of cleavable drug $[\text{E}]$, and the following formula was used:

$$\text{release rate} = -\frac{d[\text{E}]}{dt} = k[\text{HO}^-] [\text{E}] [\text{H}_2\text{O}]$$

To study the effect of relevant parameters on the release rate ($-\text{d}[\text{E}] / \text{dt}$), the release rates of PAA-ValG 15-2 (averaged over 2 months for simplification) at different pH values were used, since the relevant parameters changed with the pH value:

Figure 6.23.: Release rate of ganciclovir from PAA-ValG 15-2 at different pH.

The qualitative observation showed a clear pH-dependent release behavior, with a faster release at the initial phase with a higher pH value. These results were expected, since the pH-specific reaction constant ($k [\text{HO}^-]$), as well as the degree of hydration $[\text{H}_2\text{O}]$ increased with the pH value. Over the release time, the release rates of higher pH values were expected to decrease faster (or increase slower), since the concentration of cleavable drug $[\text{E}]$ decreased faster with higher release rates.

In an initial assumption, the pH in the microclimate was seen as identical with the surrounding pH of the buffered release medium, which was constant during release. The release rate and the concentration of cleavable drug [E] were known from the measured release profiles. The pH-specific reaction constant ($k [\text{HO}^-]$) for the release media specific pH value was taken from section 6.2.

The degree of hydration $[\text{H}_2\text{O}]$ was calculated with the following formula:

$$[\text{H}_2\text{O}] = \frac{\text{Release rate}}{k[\text{HO}^-] [\text{E}]}$$

The following degree of hydration for each time interval and pH was calculated:

Figure 6.24.: Calculated degree of hydration of PAA-ValG 15-2 at different pH.

Table 6.9: Calculated degree of hydration of PAA-ValG in numbers:

pH	Time in months					
	1 + 2	3 + 4	5 + 6	7 + 8	9 + 10	11 + 12
6.0	0.84	0.77	0.80	0.74	0.71	0.75
7.0	0.14	0.13	0.12	0.11	0.11	0.11
7.4	0.09	0.08	0.07	0.06	0.06	0.05
8.0	0.05	0.03	0.02	0.02	0.01	0.01

The calculated values for the degree of hydration were not in accordance with the previously obtained results from the tablet swelling experiments in Section 6.3, where the swelling (representing the degree of hydration) increased with the pH and time. This led to the conclusion, that the microclimate $[\text{HO}^-]$ must be different from the surrounding pH (release medium), as discussed in Section 6.4.

To estimate the pH in the microclimate, the pH-specific reaction constant ($k [\text{HO}^-]$) was calculated, using the following formula:

$$k[\text{HO}^-] = \frac{\text{Release rate}}{[\text{H}_2\text{O}] [\text{E}]}$$

The release rate and the concentration of cleavable drug $[\text{E}]$ was known from the measured release profiles. The polymer was defined to be fully hydrated, so that the degree of hydration $[\text{H}_2\text{O}]$ is 1.

Then the pH-specific reaction constant ($k [\text{HO}^-]$) was converted into the equivalent pH value, using the derived equation from Figure 6.9:

$$\text{pH} = 0.4343 \ln (k [\text{OH}^-]) + 7.4421$$

The following microclimate pH over the release of PAA-ValG was calculated:

Figure 6.25.: Calculated microclimate pH over the release of PAA-ValG 15-2.

The calculated microclimate pH qualitatively followed the expected pattern from Figure 6.22, where the samples of initial higher pH saw a greater drop, since the buffer capacity of non-conjugated PAA groups had increased towards their pKa of 5.1 - 6.2.

This plotting should not be mistaken for the actual microclimate pH, since the effect of the polymer was defined to be fully hydrated (degree of hydration = 1).

To evaluate the effect of the degree of hydration, the microclimate pH, which was determined by titration in 0.125 M NaCl (Figure 6.22) was plotted together with the microclimate pH, which was calculated from the release rate in buffered environment, with the assumption that the polymer is fully hydrated. Results gained from using an initial pH of 7.4 are shown:

Figure 6.26.: Calculated vs determined microclimate pH

Looking at both curves, the microclimate pH from the release rate was seen to be lower than the microclimate pH in an unbuffered system at the start. Since the COOH groups from the formed PAA-Val lowered the pH in an unbuffered system, this makes it impossible to achieve a lower microclimate pH in a buffered system. This meant that the difference between the minimum achievable pH and the, from the release rate calculated pH, must be caused by an incomplete hydration of the polymer, which reduced the release rate.

Based on the difference between calculated and determined microclimate pH, the degree of hydration was calculated:

Figure 6.27.: Calculated degree of hydration from the microclimate pH

The calculated values for the degree of hydration were in agreement with previously obtained results from the tablet swelling experiments in Section 6.3, where the degree of hydration increased with the pH and time.

It is theoretically not possible for the calculated degree of hydration to reach a value above 1 (since it is a factor between 0 and 1), as this means that none of the relevant ester bonds were hydrated, and that all the cleavable bonds were hydrated.

It can be explained by the buffering capacity of the release medium, which can increase the microclimate pH more, with increasing degree of hydration, resulting in a higher difference between titrated pH and calculated microclimate pH, which then appeared as higher degree of hydration.

Since the surrounding buffer can only increase the pH and the actual microclimate pH cannot be below the titrated minimum pH, the calculated degree of hydration must be seen as a maximum, which confirms that the degree of hydration played an important role in the release of ganciclovir from PAA-ValG.

6.6. Conclusion

In this chapter, the release mechanism for the release of ganciclovir from PAA-ValG was studied by analyzing the individual parameters which are the reaction constant, microclimate pH, concentration of cleavable drug, and the degree of hydration.

The reaction constant was determined on acylated valganciclovir (Ac-ValG), since its chemical structure next to the relevant ester bond was similar to PAA-ValG, without having the effects of the polymer. The reaction constant was found to be linear increasing with the hydroxide ion concentration.

The degree of hydration (penetration of hydrolyzing agent into the polymer) was studied by tablet swelling experiments and found to be increasing with time and pH.

The decrease in the microclimate pH during release, was studied via titration and found to be stronger at high pH values, and the beginning of the release.

The decrease of the concentration of cleavable drug was calculated from the release profiles.

The combination of reaction constant, microclimate pH, concentration of cleavable drug, and the degree of hydration led to a formula which described the release rate accurately.

7. Evaluation of the *in vitro* drug release testing

7.1. Introduction

The release properties of every sustained-release drug formulation are beside the assay, degradation and impurity determination most relevant. The *in vitro* release behavior helps to predict the *in vivo* release behavior, which is important to help determine the amount of drug substance released at every time point during application. Besides testing under physiological conditions (e.g. PBS pH 7.4), *in vitro* release testing under non-physiological conditions can also be used to understand the mechanism of release.

7.2. Drug Release Testing Method

The methodology can affect the results significantly, especially so during release testing. The commonly used drug release testing methods for polymeric particulate systems are classified into three different methods: the **Continuous Flow method**, the **Dialysis method**, and the **Sample and Separate method** [71].

It is important to mention that a drug release from polymer drug conjugates also involves the hydrolysis of the labile bond, so that the term “dissolution testing” does not describe the process, even though the methodology for release and dissolution testing can be quite similar, provided that the polymer drug conjugate is seen as a particle.

7.2.1. Continuous Flow Method

When using the **continuous flow method**, the sample is placed inside a flow-through cell which is continuously flushed with pre-heated release media. Based on the mode of operation, the release media is either circulated (closed loop) or replaced with fresh media (open loop).

Since there is no “**flow-through cell dissolution apparatus (USP 4)**” available in the used laboratory, the **continuous flow method** was not considered for release testing. Even if such an equipment was available, the number of samples which could be studied over a long period of time would be very limited. That is also the reason why this method is less commonly adopted for long term release testing in academia.

7.2.2. Dialysis Method

When using the **dialysis method**, the sample and some of the release media are placed inside a dialysis bag which is then placed inside a vessel with more release media and incubated at the desired temperature. The release media is replaced fully (in rare cases partially) at the desired time points and analyzed, while the sample containing release media inside the dialysis bag cannot be replaced.

The molecular weight cut-off of the dialysis bag has to be below the lowest molecular weight of the fully released polymer drug conjugate (to avoid escaping of polymer), but above the molecular weight of the active ingredient (to allow the drug to diffuse out).

7.2.3. Sample and Separate Method

The **Sample and Separate method** is commonly used in the pharmaceutical industry to perform dissolution testing on solid oral dosage forms such as tablets and capsules. The sample is placed in temperature-controlled release media, either inside a **rotating basket (USP 1)** or directly inside the release media which is stirred at a specified speed with a **rotating paddle (USP 2)**. At desired time points, an aliquot is removed (sample) and filtered (separate) to remove undissolved drug and excipients, which would further dissolve and influence the result.

In case of polymer drug conjugates which are fully or partially soluble, the filtration would have to be performed on a molecular level e.g. by using dialysis membranes. Instead the further release can be quenched e.g. by changing pH or temperature and the separation can be performed inside a HPLC system prior to detecting via UV-light.

If the drug release from polymer drug conjugates is significantly more dependent on the chemical degradation/ hydrolysis of the linker, than on dissolution/ diffusion, no stirring at all might be necessary to obtain reproducible results.

A replacement of the removed (sampled) release media with fresh release media is usually only performed, if the volume of all samples exceeds more than two percent of the total volume release media and might lead to false results if the carrier/ polymer drug conjugate is homogenous distributed in the release media at the time of sampling.

7.2.4. Dialysis vs. Sample and Separate Method

This leaves the **Dialysis method** and the **Sample and Separate method** as options, which are performed in different variations, as shown in the following illustration:

Figure 7.1.: Variables in commonly used drug release testing methods

The dialysis method is expected to be performed with replacement of the entire release media (Vm) at each timepoint, except for the volume inside the dialysis bag (Vd), where else the Sample and separate method is performed in a way that the total volume of all samples (Vs) does not exceed 2% of the total volume (Vm) and no media (Vr) replaced.

For diffusion-based systems like liposomes or hydrogels, the dialysis method is the preferred method as it is difficult to stop the release after sampling and the injection into a HPLC system with organic solvent environment could release them fully.

In degradation/ hydrolysis-based systems like polymer drug conjugates, the release can be quenched by changing the pH, so that even the contact with organic solvents won't change the release for the short duration of a HPLC analysis. The same method was also performed by Stefanidis and Brandel when performing experiments which could also be described as: "determining the release of ganciclovir from valganciclovir" [61].

For PAA-ValG, the Sample and Separate method was chosen, after determining the stability of ganciclovir and the ability to quench the release:

The stability of the studied drug in the release media is more relevant in the sample and separate method, since the media is not exchanged until the end of the release. A volumetric flask with ganciclovir solution in PBS pH 7.4 was stored at 60 °C and analyzed regularly to determine the stability/ change in concentration.

To determine the ability to quench the release of ganciclovir from PAA-ValG, samples were re-analyzed after one year of storage (adjusted to pH 3 in the refrigerator 2-8 °C) and the change in the released amount of ganciclovir was subtracted from the total amount of PAA-ValG. The following results were obtained:

Figure 7.2.: Stability of ganciclovir during release and PAA-ValG after sampling

Ganciclovir in PBS pH 7.4 at 60 °C was showed to be very stable, allowing for release testing to be conducted over a long period of time.

PAA-ValG in PBS (adjusted to pH 3) at 2-8 °C was shown to be very stable, but the samples were measured on the same day or latest in the same week of the sampling, with the option of re-analysis.

7.3. Release medium optimization

A release medium with similar properties as vitreous humor can enhance the predictive capability of the release testing. Even though there are different protocols for the preparation of simulated vitreous humor available in literature, the way of simulating biological fluids is highly dependent on the intended use. This made the development of a project specific release medium necessary. Herein, the main properties and ingredients of vitreous humor were considered:

Table 7.1: Properties of vitreous humor

Category	Name	Value (average)	Source
Physical properties	Volume	3.9 ml	[72]
	pH	7.40-7.52	[72]
	Osmolality	289.5 mOsm/kg	[73]
	Dynamic viscosity	1.6 cP	[72]
Cationic solutes	Sodium	146.7 mmol/l	[73]
	Potassium	5.73 mmol/l	[73]
	Calcium	1.13 mmol/l	[73]
	Magnesium	0.90 mmol/l	[73]
Anionic solutes	Chloride	121.6 mmol/l	[73]
	Bicarbonate	19.7–49.2 mmol/l	[72]
	Pyruvic acid	8.3 mmol/l	[72]
	Lactate	3.97 mmol/l	[73]
	Ascorbic acid	2.04 mmol/l	[72]
	Phosphate	0.33–1.1 mmol/l	[72]
	Citrate	0.10 mmol/l	[72]
Neutral solutes	Urea	4.0–28.6 mmol/l	[72]
	Glucose	2.97 mmol/l	[73]
Macromolecular solutes	Total protein content	280–1360 mg/l	[72]
	Hyaluronan	42–400 mg/l	[72]
	Collagen	300 mg/l	[74]
	Albumin	293 mg/l	[72]
	Enzymes / Esterase	present	[75]

7.3.1. Initial release medium

Release Media (PBS pH 7.4)

The initial release experiments were performed with commercially available Phosphate Buffered Saline (PBS) pH 7.4 from Sigma Aldrich, which consisted of 0.01 M phosphate buffer and 0.0027 M KCl and 0.137 M NaCl.

The frequent use of PBS for release testing in literature and its similar properties to vitreous humor (pH and salinity) made PBS a good starting point for the evaluation of relevant parameters for the optimization of the release medium.

Sample preparation:

Unless specified otherwise, 25 mg of PAA-ValG 15-2 were weighed into a 250 ml volumetric flask and filled up with release medium to the mark. The volumetric flask was shaken at 50 rpm in a 37 °C environment. At each time point, 500 µl of the clear supernatant (in case that PAA-ValG was not/ not yet dissolved) were removed and diluted with 50 µl of 0.15 M H₃PO₄ into HPLC vials. The samples were stored in the refrigerator until HPLC analysis.

Calculation*:

$$\% \text{ Released} = 100\% \frac{C_{Gan \text{ released}}}{C_{Gan \text{ in sample}}}$$

*The exact sample weight, as well as dilutions were considered.

7.3.2. pH-Value

The pH value is expected to be most important of all listed properties, since the release mechanism of PAA-ValG is based on the hydrolysis of the ester bond between ganciclovir and the L-valine linker, which was shown to be proportional to the hydroxyl ion concentration in the physiological pH range (section 6.2).

Furthermore, the pH has a significant influence on the degree of hydration, since there were multiple functional groups inside PAA-ValG which can be charged, depending on their pK_a values, as shown in section 6.3.

To determine the pH dependence of the ganciclovir release PAA-ValG 15-2 was released in pH-adjusted PBS of pH 6.0, 7.0, 7.4 and 8.0. The pH was monitored over the entire release and corrected where necessary:

Figure 7.3.: Release of ganciclovir from PAA-ValG 15-2 different pH values.

A high pH dependence of the ganciclovir release, as well as the need for multiple pH corrections (pH dropped during release) were observed.

The preferred pH for further release experiments was set to 7.4 (7.40 to 7.44), because it was within reported pH of vitreous (7.40-7.52) [3] and it was still within the pH of blood (7.35-7.45) [76], which helped to predict the release behavior of PAA-ValG in other medical applications of ganciclovir.

7.3.3. Ionic composition / Osmolarity

The ionic composition of the medium is expected to have an effect on the release, since the pKa of aqueous PAA solution was reported to decrease when NaCl is added [77]. This does result in a change of the **degree of dissociation (ionization) α** at constant pH, which would then result in a change of hydrophobicity/ degree of hydration.

In an initial experiment, 5 samples of PAA-ValG 15-2 were released in PBS of different concentrations reaching from 0.5 to 1.5 times of the physiological osmolarity. The pH was monitored over the entire release and corrected to pH 7.4 if necessary.

Figure 7.4.: Release of ganciclovir from PAA-ValG15-2 at different pH

Even though the release profiles showed a very similar pattern with a maximum relative standard deviation of 10% over all tested PBS concentrations, it seems that the lower PBS concentrations are releasing ganciclovir at a slower rate.

This can be explained, since at a lower salt concentration (higher pKa, less groups charged) the degree of hydration was expected to be lower than at higher salt concentration (lower pKa, more groups charged).

To confirm this observation, the quantitative effect of the ionic concentration on the pKa of PAA-groups was studied, making use of the Henderson-Hasselbalch equation:

$$pH = pKa + \log\left(\frac{\alpha}{1 - \alpha}\right)$$

which expresses, that the pKa value of a substance is equivalent to the pH of the solution when as many groups are protonated as deprotonated ($\alpha = 0.5$).

PAA was used, since degradation changes α of COOH-groups in PAA-ValG.

To determine the total number of COOH groups in the used, commercially available PAA solution, an aliquot was titrated with $c(\text{NaOH}) = 0.1000 \text{ mol/l}$ (freshly opened, commercially available alkaline standard solution from Sigma Aldrich):

Figure 7.5.: Titration of PAA (30 kDa) against $c(\text{NaOH}) = 0.1 \text{ mol/l}$

The equivalent point showed the volume of $c(\text{NaOH}) = 0.1 \text{ mol/l}$ which was necessary to deprotonate all COOH-groups ($\alpha = 1$).

In this example 217 mg of PAA (30 kDa) solution were titrated with 9.10 ml $c(\text{NaOH}) = 0.1000 \text{ mol/l}$ which was equivalent to 29.78 % (w/w) PAA in the solution. That concentration was equivalent to 99.3 % of the declared 30 % PAA (w/w).

Result: 20.97 ml NaOH was needed to adjust 1 g of the PAA (30 kDa) to $\alpha = 0.5$.

The following pKa values in dependence of concentration and ionic strength were measured:

Figure 7.6.: Effect of concentration and osmolality on the pKa of PAA (30KDa)

It was observed that the pKa was dependent on the concentration of NaCl, but not on the concentration of PAA, with two exceptions:

PAA-solutions in pure water, showed a concentration dependence which was reported by Arnold [7] and was expected to be caused due to the effect of the PAA and containing Na-ions on the solutions which is insignificant in the NaCl containing solutions.

It was also observed that the 0.125 mg/ml PAA in 1 M and 2 M NaCl showed a higher pH than solutions with more PAA. This was expected to be caused by the lower buffer capacity of PAA towards the NaCl solutions of pH 5.5 (1 M and 2M NaCl).

Based on the results, the pKa values in the range of 8 to 0.5 mg/ml PAA in all NaCl containing solutions were seen as representative and averaged to determine the mathematical relationship between NaCL concentration and pKa:

Figure 7.7: Effect of NaCl on the pKa of PAA (30KDa)

The following relationship was found:

$$pKa = 4.9144 C_{NaCl}^{-0.043}$$

Table 7.2: Effect of NaCl on the pKa of PAA (30KDa) in numbers:

NaCl (mol/L)	pKa (measured)	SD pKa (measured)	pKa (calculated)	Deviation pKa calculated - pKa measured
0.0625	5.49	0.02	5.54	0.04
0.125	5.39	0.01	5.37	-0.01
0.25	5.25	0.01	5.22	-0.03
0.50	5.09	0.01	5.06	-0.03
0.75	4.99	0.01	4.98	-0.02
1.00	4.92	0.01	4.91	0.00
2.00	4.75	0.01	4.77	0.02
3.00	4.67	0.00	4.69	0.02

The maximum standard deviation over all averaged PAA-concentrations was 0.02 pKa units and the maximum deviation between calculated curve and average value was 0.04 pKa units.

To determine the effect of the cationic composition of the release medium, represented by the Na^+/K^+ ratio, which were the main cations in vitreous humor, the effect of K^+ on the pKa of PAA(30KDa) was studied. Therefore, solutions with a final concentration of 4 mg/ml PAA(30KDa) and varying concentrations of NaCl, KCl or both were prepared. The relatively high concentration of PAA was chosen to keep the buffer capacity high, so that differences in the pH of the used NaCl or KCl solutions will not affect the results.

The following pKa values were measured:

Figure 7.8.: Effect of NaCl or KCl on the pKa of PAA (30KDa)

Since the used PAA stock solution was adjusted to $\alpha = 0.5$ with NaOH for all samples to avoid a systematic error due to differences in the molarity of NaOH and KOH, the additional Na^+ content of 0.02775 mol/L was considered in the Na^+/K^+ ratio:

Table 7.3: Mole fraction of Na^+ in the measured pKa curves:

Mole fraction of Na^+	Concentration of NaCl or KCl				
	0.125M	0.25M	0.50M	0.75M	1.0M
NaCl	1.00	1.00	1.00	1.00	1.00
Na/KCl (50%)	0.59	0.55	0.53	0.52	0.51
KCl	0.18	0.10	0.05	0.04	0.03

To determine the effect of K^+ alone, as well as Na^+/K^+ ratio dependent pKa, the mol fraction of Na^+ was plotted against the measured pKa values:

Figure 7.9.: Effect of Na^+ to K^+ ratio on the pKa of PAA (30KDa)

A linear relationship between the pKa of PAA and the mole fraction of Na^+ in all Na/KCL containing solutions was observed.

Table 7.4: Effect of Na^+/K^+ on the pKa of PAA (30KDa) in numbers:

Na/KCl (mol/L)	Slope	Intercept	R ²
0.125	-0.0489	5.4089	1.0000
0.25	-0.0889	5.3089	1.0000
0.50	-0.1583	5.2100	0.9990
0.75	-0.2074	5.1607	0.9977
1.00	-0.2364	5.1247	0.9996

The intercept of this curves is representing the pKa of only K^+ -containing solutions.

The relationship between KCl concentration and pKa was determined:

Figure 7.10.: Effect of KCl on the pKa of PAA (30KDa)

The following relationship was found:

$$pKa = 5.1216 C_{KCl}^{-0.026}$$

Combining the formulas for the effect of NaCl (figure 7.7) and KCl on the pKa of PAA (30KDa) results in the following equation:

$$pKa = (1 - X_{Na^+}) 5.1216 (C_{NaCl} + C_{KCl})^{-0.026} + X_{Na^+} 4.9144 (C_{NaCl} + C_{KCl})^{-0.043}$$

Table 7.5: Effect of Na/KCl on the pKa of PAA (30KDa) in numbers:

NaCl + KCl (mol/L)	Mole fraction Na ⁺	pKa (measured)	pKa (calculated)	Deviation pKa calculated - pKa measured
0.125	0.59	5.38	5.39	0.01
0.125	0.18	5.40	5.40	0.00
0.25	0.55	5.26	5.26	0.00
0.25	0.10	5.30	5.30	0.00
0.50	0.53	5.13	5.13	0.00
0.50	0.05	5.20	5.21	0.01
0.75	0.52	5.06	5.06	0.00
0.75	0.04	5.15	5.15	0.00
1.00	0.51	5.00	5.02	0.02
1.00	0.03	5.12	5.12	0.00

The maximum deviation between calculated pKa and measured pKa was 0.02 pKa units.

Since the ionic composition (e.g. NaCl concentration) was directly proportional to the osmolarity of a solution, it had to be verified, if the pKa dependence of PAA(30KDa) was dependent on the ionic strength only, or also on the osmolarity which can be changed by neutral solutes.

To determine the effect of the osmolarity on the pKa of PAA(30KDa), solutions with a final concentration of 4 mg/ml PAA(30KDa) and varying concentrations of NaCl, and glucose were prepared. The relatively high concentration of PAA was chosen to keep the buffer capacity high, so that differences in the pH of the used NaCl and Glucose solutions will not affect the results. The following pKa values were measured:

Figure 7.11.: Effect of the osmolarity on the pKa of PAA (30KDa)

There was no significant effect of the osmolarity on the pKa of PAA (30KDa) measurable. The maximum standard deviation per NaCl concentration was 0.01 pKa units.

Based on this results, only ionic compounds (and not osmolarity) were considered when preparing the optimized release medium.

7.3.4. Effect of multiple charged ions

It is also known that the presence of multiple charged ions (e.g. Ca^{2+} or Mg^{2+}) can lead to ionic crosslinking of polymers with multiple negative charges. One commonly known example was the crosslinking of Alginate with Ca^{2+} . Bouhadir et.al. reported that the release of the drug daunomycin from poly (aldehyde guluronate) hydrogels changed from 100 % release in 4 weeks to 40 % release in 6 weeks, when Ca^{2+} was added to the release medium [78].

To determine if the same effect applies to the PAA-backbone of PAA-ValG when in contact with Ca^{2+} or Mg^{2+} from vitreous humor, release testing in PBS with physiological concentrations of Ca^{2+} (1.13 mmol/L CaCl_2), was performed. The pH was monitored over the entire release and corrected to pH 7.4 where necessary:

Figure 7.12.: Release of ganciclovir from PAA-ValG15-2 in presence of Ca^{2+} .

Even though the PAA-ValG in calcium containing release samples did never become fully dissolved/ clear as Ca-free samples, over the release time of 12 months, the release profiles did not differ significantly. The deviation of Ca-containing release profiles was below the standard deviation of Ca-free PBS (n=6) at all points.

Based on these results, no Ca^{2+} or Mg^{2+} was placed in the optimized release medium.

7.3.5. Volume of release medium (sink conditions)

Even though the volume of vitreous humor in the eyes was reported to be 3.9 ml [72], there was diffusion/ clearing processes ongoing, so the drug release was not expected to be limited by the volume of vitreous humor or the solubility of ganciclovir. This made sink conditions preferable for the release testing, which is defined as a volume of release medium, that can dissolved at least 10 times that of the present drug concentration [79].

Since the solubility of ganciclovir was reported to be 4.3 mg/ml under physiological conditions [3] and the assay of PAA-ValG was 42.0 % $\pm$ 1.1 % ganciclovir (w/w), a concentration of equivalent or less than 1.0 mg / ml PAA-ValG in the release medium were calculated to fulfill sink conditions.

To evaluate if there were any effects of drug concentrations on the release testing, 25 mg PAA-ValG each were released in either 50 ml or 250 ml PBS pH 7.4. The pH was monitored over the entire release and corrected to pH 7.4 where necessary:

Figure 7.13.: Volume dependent release of ganciclovir from PAA-ValG 15-2.

The difference between both concentrations was below the standard deviation of the 0.1 mg/ml PAA-ValG sample in PBS pH 7.4 (n=6) at all points. Even though the release profiles were very similar, the higher buffer to PAA-ValG ratio in the 0.1 mg/ml sample was preferred, since less pH adjustments were necessary.

The buffer capacity was considered for the optimized release medium.

7.3.5. Effect of macromolecules / enzymes

Even though the protein binding of ganciclovir was reported to be only 1 to 2 % [3] the effect of enzymes from biological fluids can be significant.

For example, Cavallaro et. al. reported a 7 to 8 times faster release of ganciclovir from a polymer drug conjugate in human plasma compared to buffer pH 7.4 [12]. The effects were expected to be caused by esterase in human plasma, which was able to access ester-linked polymer drug conjugate in the soluble state.

On the other hand, D'Souza et. al. reported that enzymes cannot enter polymer drug conjugates in the solid state (e.g. hydrogels, micelles) [13].

Even though Esterase was reported to be present in human vitreous [6] and PAA-ValG also belonged to the group of ester-linked polymer drug conjugates, an enzymatic release of ganciclovir is uncertain, due to two reasons:

1. The state of PAA-ValG (soluble, solid, gel) was dependent on the concentration and changes with the release of ganciclovir.
2. The concentration and activity of esterase that PAA-ValG would be exposed under *in vivo* conditions is unknown.

This led to the conclusion that the effect of enzymes and other biological ingredients should not be part of the *in vitro* release testing and should be evaluated in animal studies or possibly *ex vivo* conditions.

Enzymes and other biological ingredients were not considered in the release medium.

7.3.6. Improved release medium (PBN pH 7.4)

Considering the parameters affecting the release, an improved release medium called PBN with a higher buffer capacity and 0.05 % (w/v) NaN_3 to avoid contamination was prepared as described in material and methods.

The ganciclovir release from PAA-ValG in PBS pH 7.4 and PBN pH 7.4 was compared:

Figure 7.14.: Media dependent release of ganciclovir from PAA-ValG 15-2

The difference between PBS and PBN was below the standard deviation of the sample in PBS pH 7.4 (n=6) at all points. No pH adjustment had to be made for PBN pH 7.4.

The relative standard deviation for the release samples in PBN (n=6) was improved:

Figure 7.15: Media dependent release of ganciclovir from PAA-ValG 15-2.

7.4. Conclusion

In this chapter the different release testing methods were discussed. Based on the stability of ganciclovir under release conditions, as well as the stability of PAA-ValG when quenching the release by pH adjustment, the use of the sample and a separate method was recommended.

Furthermore, the composition of the release media was discussed based on the reported composition of vitreous humor from literature. The relevant physical properties, as well as the qualitative and quantitative composition of the release media, was evaluated. It was found that the pH value of the release media had the highest effect on the observed release profiles, followed by the ionic composition (as NaCl concentration). The ratio of Na^+ to K^+ has an effect on the release profile, but was negligible, since less than 1 % of all ions in VH were K^+ . Similar outcome was expected to happen with Ca^{2+} , but experiments were not able to show a significant difference in the release profiles. The presence of biological components was not considered for *in vitro* release testing. The evaluation of the drug/ conjugate concentration in release media showed that sink conditions were obtained with concentrations of 0.5 mg/ml PAA-ValG, which was equivalent to 0.2 mg/ml ganciclovir.

Based on the obtained results, it was shown that the use of PBS pH 7.4 as well as the improved release medium PBN pH 7.4 (which was basically PBS with higher buffer capacity and NaN_3 as preservative) were both suitable to simulate the conditions of vitreous humor *in vitro* release testing.

Overall, the chosen release testing method, in combination with the chosen release medium should be able to simulate the non-enzymatic release of PAA-ValG after intravitreal injections well.

8. Conclusions

In this study, a ganciclovir-loaded polymer drug conjugate for the long-term sustained release for the treatment of CMV retinitis was studied. By using a simple polymer structure, consisting of polyacrylic acid (PAA) as polymer backbone, L-valine as linker and ganciclovir as drug, a drug loading of 42 % ganciclovir (w/w) was achieved, which was necessary to reduce the number of intravitreal injections. Since the synthesis was performed by EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) coupling of valganciclovir (L-valine ester prodrug of ganciclovir) to polyacrylic acid in aqueous buffer solution, where all substances except for the polymer drug conjugate (PAA-ValG) were soluble, a high product quality (< 0.5 % free drug and < 0.2 % impurities) was achieved. The purification process used where the product was washed at pH 3 also ensured a high storage stability, since the relevant ester bond was known to be most stable at an acidic pH.

A long-term sustained release over more than 1 year was observed, when performing *in vitro* release testing in a release medium which has all relevant properties of vitreous humor, except for its biological components. This release was much longer than all other ganciclovir-releasing systems reported [6-12]. Though *ex vivo* release testing with pig-vitreous showed no significant change in the release rate when PAA-ValG was in the solid state, the release rate was observed to be 8 times higher when PAA-ValG was in the soluble state. Since PAA-ValG was observed to change from the solid to soluble state during release, the *in vivo* release was therefore expected to be faster, but also more constant than the *in vitro* release. In the best case scenario, one injection of around 11 mg PAA-ValG (depending on the exact drug content), equivalent to 4.5 mg ganciclovir, will be sufficient for 6 to 8 months of treatment, as it is in the ganciclovir-releasing implant ‘Vitrasert’ (which was later discontinued) [3, 14], albeit with less side effects, due to its injectability.

Taking an overall view, the high drug loading, purity, stability, long-term sustained release and predicted properties from literature makes PAA-ValG an excellent candidate for *in vivo* studies, with the goal to control cytomegalovirus retinitis with fewer intravitreal injections as per the current treatment.

As a major part of this work, the release mechanism for the *in vitro* release was studied. For this purpose, acylated valganciclovir (Ac-ValG) was synthesized and its reaction constants for the pseudo-first-order release rate of Ac-ValG were determined. This was necessary to study the ester hydrolysis without the effects of the polymer, since its chemical structure or Ac-ValG next to relevant ester bond was similar to that of PAA-ValG. The reaction constant for the ester hydrolysis was found to be pH dependent and linear increasing with the hydroxide ion concentration in the release medium. Since PAA-ValG did not follow the same release profile, the penetration of hydrolyzing agent (water or hydroxide ions) into the polymer (degree of hydration) was studied by tablet swelling experiments, as previously performed by Pitt and Shah [15] and found to be time and pH dependent. Furthermore, the decrease in the microclimate pH during release, as it was known from other biodegradable polymers [16], was studied via titration and found to be stronger at high pH values, and the beginning of the release. By combining of reaction constant, microclimate pH, concentration of cleavable drug and the degree of hydration in one formula, release rate was explained.

9. Future Work

9.1. *In vivo* testing for the treatment of CMV retinitis with PAA-ValG

While all characterization and release studies in this thesis were conducted *in vitro* or *ex vivo*, meaningful data can be obtained from *in vivo* studies. A basic *in vivo* study consists of pharmacokinetic, pharmacodynamics and toxicity:

Pharmacokinetic studies can show the effect of the sustained release in terms of drug concentrations in vitreous, serum and tissues of interest.

Pharmacodynamic studies are an indicator for the regression of the disease. Since the ganciclovir eluting intravitreal implant **Vitrasert** was approved for the treatment of CMV retinitis, it is known that *in vivo* release rates of 4.5 mg ganciclovir over a period of 6 to 8 months [14] are sufficient to treat CMV retinitis. Therefore, the results from the pharmacokinetic study can be used to predict pharmacodynamics without the use of an animal model of CMV retinitis.

Ocular toxicity is commonly investigated by using electroretinography (ERG). It provides information about outer and inner retinal function before and after drug application. *In vivo* cytotoxicity can also be detected with histological analysis by hematoxylin and eosin staining verifying any retinal necrosis, morphological changes in the eye and infiltration of inflammatory cells after treatment.

Pharmacokinetic studies, combined with thorough examination of retinal function should be sufficient to show, if PAA-ValG can be used to treat CMV retinitis in a save manner.

9.1.1. Protocol for pharmacokinetic studies of intravitreal PAA-ValG

48 rabbits, randomly distributed into 12 experimental groups of 4 animals per group, will be needed for the *in vivo* studies of PAA-ValG. Rabbits will be anesthetized by intramuscular injection of ketamine hydrochloride and xylazine hydrochloride before intravitreal injection. The topical anesthesia will be achieved using 1% lignocaine hydrochloride (50 mg/ml). PAA-ValG, equivalent to 4.5 mg ganciclovir, dissolved in diluted aqueous NaOH to a total volume of 100 μ l, having pH 7.4, will be administered with a single intravitreal injection to the right eye using 30-gauge needle and the left eye serve as a control. Four rabbits will be sacrificed at each time points (1 hr, 3 hrs, 24 hrs, 7 days, 14 days, 30 days, 60 days, 90 days, 120 days and 180 days). Both right and left eyes will be enucleated and frozen immediately and store at -80°C. The eyes will be thawed, and various ocular tissues will be dissected and subjected for HPLC analysis. For systemic pharmacokinetic studies, n=4 animals will be used. The blood samples will be collected through marginal ear vein before sacrifice at 1 hr, 3 hrs, 24 hrs, 7 days, 14 days, 30 days, 60 days, 90 days, 120 days and 180 days. On the day of analysis, the tissues and blood samples will be processed as per reported standard protocol.

9.1.2. Protocol for safety studies of intravitreal PAA-ValG

To evaluate the safety of intravitreal PAA-ValG, 4 rabbits will be injected intravitreally with PAA-ValG and after 180 days, the eye will be enucleated and will be subjected for histology studies and will be compared with the control eyes.

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