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New Studies on Protein Loaded VPG as Controlled Release Systems

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FOR MY FAMILY

Chapter 1 General Introduction

1.1 Protein therapeutics

Proteins are one of the most important biologic macromolecules, and therapeutic proteins play a crucial role in the treatment of various diseases. In 1922, the discovery of insulin by Frederick Banting and John Macleod, marked a major breakthrough in medicine and therapy in patients with diabetes[1]. In 1975, the basis for clinical applications of monoclonal antibodies was set by the generation of continuous cultures of hybridoma cells by Georges J. F. Köhler and César Milstein (Figure 1-1, A) [2,3]. With further development of biotechnology and biomedicine, the recombinant protein technology was built in the 1980s, and purified protein products with special functions were established by bio-engineering in industry[4]. Protein therapeutics have become a powerful treatment method, and nowadays lots of proteins and peptides have been approved for clinical use by the US Food and Drug Administration (FDA)[5-7].

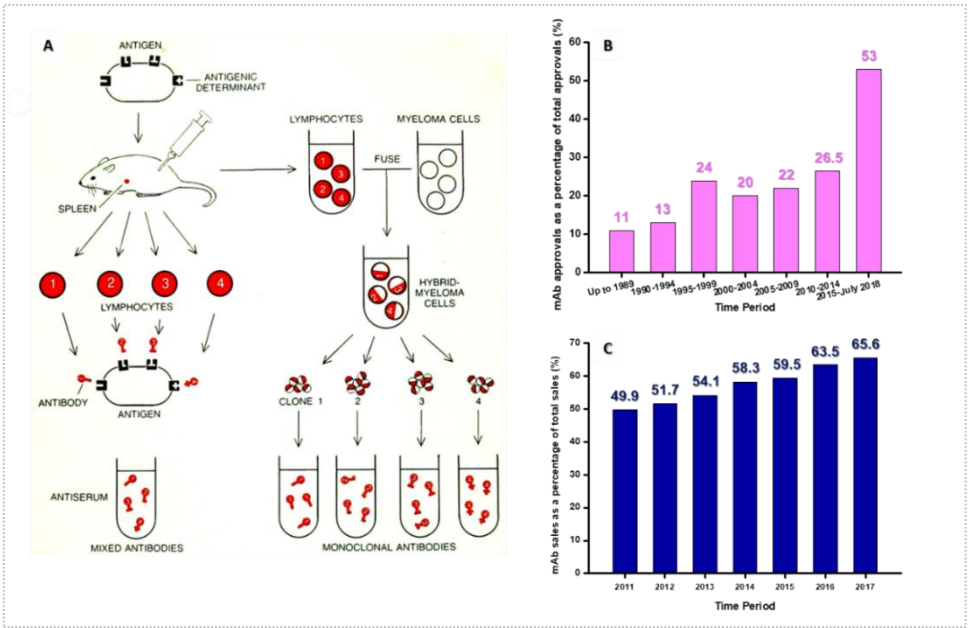

Figure 1-1 The development of monoclonal antibody production. (A) preparation of monoclonal antibodies[8]. (B) mAbs approved in the indicated periods, expressed as a percentage of total biopharmaceuticals approved in the same time period. (C) mAbs global annual sales value expressed as a percentage of total biopharmaceutical global sales for the indicated years[9].

Since the FDA approved the first human recombinant protein drug insulin in 1982, many proteins have been developed as therapeutics[10]. Most protein based drugs are used in therapy for serious diseases such as diabetes, cancer or autoimmune diseases[11]. The available data also shows an increasing dominance of mAbs within the universe of

biopharmaceutical approvals. (Figure 1-1, B C)[9]. The top ten selling biopharmaceuticals together generated sales of $80.2 billion, which is almost 44% of total biopharmaceutical product revenues in 2017[9]. The global market for pharmaceuticals in 2018 was $1.2 trillion, and 11 of the Top 15 Best-Selling Drugs of 2018 were protein drugs[12].

Protein therapeutics have several advantages over most small-molecule drugs[6]. Proteins are highly specific and therefore there is often fewer potential to interfere with normal biological processes and cause side effects. Because the body naturally produces many of the proteins that are used as therapeutics, these agents are often well tolerated and are less likely to elicit immune responses. For diseases in which a gene is mutated or deleted, protein therapeutics can provide effective replacement treatment without the need for gene therapy, which is not currently available for most genetic disorders. The clinical development and FDA approval time of protein the therapeutics may be faster than that of small-molecule drugs. Last, because proteins are unique in form and function, companies can obtain far-reaching patent protection for protein therapeutics[4]. The protein therapeutics are also highly investigated in drug research and development fields of this century. By the help of new protein therapeutics, people can overcome many diseases and extend human life[6,13,14].

1.2 Challenges of protein drug formulations

Although many successful examples have put into clinical application, there are still some challenges in future research and development. First, as macromolecules, proteins have a highly specific structure with both hydrophilic and hydrophobic properties, hence the protein solubility, route of administration, distribution and stability are all factors that can hinder the successful application of a protein therapy. Second, the body may initiate some immune response against the therapeutic protein. In some cases, these immune responses can neutralize the protein and can even cause a very harmful reaction in the patient. Third, for a protein to be physiologically active, post-translational modifications such as glycosylation, phosphorylation, and proteolytic cleavage are often required. Last, the required costs involved in developing protein therapies are still too expensive for small pharma companies or institutes to successfully enter the market. This also means some uncertain business risk for investors [14-16].

Protein stability and immunogenicity are two main challenges to the clinical success of novel protein drug delivery systems[17].If formulations of proteins can be improved step by step in terms of solubility, route of administration, distribution and stability, there will be more methods to overcome therapeutically challenges in the future, and also more opportunities to provide advanced protein therapeutics for clinical therapy [5,6].

1.3 Development of novel protein drug formulations

Despite tremendous efforts, parenteral delivery still remains the major mode of administration for protein and peptide therapeutics[18]. Several approaches have been tried in the past to improve protein and peptide *in vitro* /*in vivo* stability and performance. Approaches may be broadly categorized as chemical modification and colloidal delivery systems. In the following, we will discuss various chemical approaches such as Half-life extension (HLE) technology, which includes PEGylation, hyperglycosylation, mannosylation, and novel delivery systems including microparticles, nanoparticles, liposomes, carbon nanotubes and micelles for improving protein and peptide delivery[18].

1.3.1 Half-life extension (HLE) technology

1.3.1.1 Preparation of PEGylated proteins

Poly ethylene glycol (PEG) is generally known as a hydrophilic, non-toxic, non-immunogenic and non-antigenic polymer. PEGylation is generally regarded as the superior approach used to increase the protein's molecular weight, which effectively increases the hydrodynamic radius of the protein molecule, and at the same time creates "stealth" molecules or drug particles, which reduces nonspecific cellular uptake by the mononuclear phagocyte system (MPS) and modulates the circulation half-life of drug molecules[19,20]. PEGylation refers to the attachment of a PEG moiety covalently to the surface of the protein or peptide through a reactive group such as amines, thiols or carboxylic acids[20]. Several PEGylated proteins are commercially available such as Adagen®, Oncaspar®, PEGIntron ®, PEGASYS, Neulasta®, Somavert®, Mircera® and Cimzia® (Table 1-1)[18].

Brand	Active substance	Company	Approval
Marketed PEGylated proteins			
Adagen®	Adenosine deaminase	Enzon	1990
Oncaspar®	Asparginase	Enzon	1994
PEG-INTRON®	Interferon α-2b	Schering-Plough	2000
Pegasys®	Interferon α-2a	Roche	2001
Neulasta®	G-CSF	Amgen	2002
Somavert®	GHA	Pfizer	2003
Macugen®	Anti-VEGF aptamer	Eyetech/Pfizer	2004
Mircera®	EPO	Roche	2007
Cimzia®	Anti-tumor necrosis factor Fab'	Nektar/UCB	2009
Krystexxa®	Uricase	Savient Pharmaceutical	2010
PEGylated drug pipeline			
Etirinotecan pegol	Irinotecan	Nektar	Phase III
Naloxegol	Naloxol	Nektar/AstraZeneca	Phase III
NKTR-181/192	Opioid analgesic	Nektar	Phase I
BAX-855	Recombinant factor VIII	Baxter	Phase I
ENZ-2208	7-Ethyl-10-Hydroxycamptothecin	Enzon	Phase II
ARX201	hGH	Ambrx/Merck Serono	Phase III

G-CSF, granulocyte-colony stimulating factor; GHA, growth hormone antagonist; VEGF, vascular endothelial growth factor; EPO, erythropoietin; hGH, human growth hormone.

Table 1-1 PEGylated drugs[21].

1.3.1.2 Hyperglycosylation

New biodegradable and biocompatible polymers are in clinical development such as dextrin [22], hydroxyethylstarch (HES) [5], polyacetal Fleximer ® [23], and polysialic acid [20,21]. The hyperglycosylation can improve pharmacokinetic behavior of therapeutic proteins like PEGylation, which can increase the *in vivo* biological activity and half-life[20]. Similar to PEGylation, it reduces interactions with antigen presenting cells, leading to prolonged systemic circulation and reduced immunogenicity. The main advantage of hyperglycosylation over PEGylation is the biodegradable nature of carbohydrates compared to the non-biodegradable PEG[18]. The HESylation® is one of promising paths of HLE technology, which significates coupling proteins to hydroxyethyl starch (HES). In the work of Robert Liebner, the site-specific HESylated anakinra was been modified, and the pharmacokinetics of the protein exhibited a 6.5-fold increase in half-life, and a 45-fold increase in AUC [5].

1.3.2 Novel delivery systems for protein drug

With further development of material and biomedicine over several years, lots of microscale and nanoscale delivery systems have been built, and "Smart" bio-responsive materials have been invented, which are sensitive to biological signals or to pathological

states, and interact with them[24]. They are powerful therapeutic platforms for the development of next-generation precision medications, and those delivery systems were also brought to the field of protein drug formulation to enhance therapeutic performance or decrease toxicity and side effects.

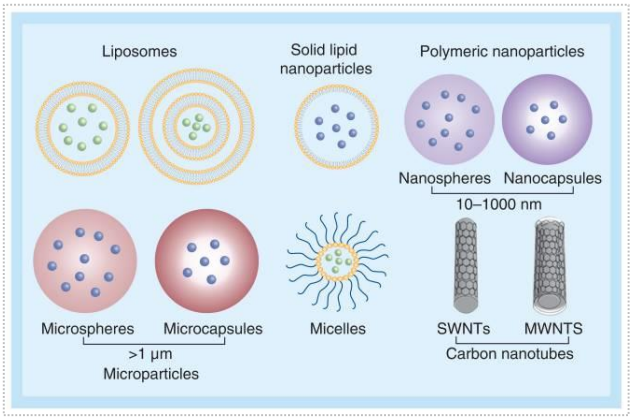

Figure 1-2. Colloidal carriers for protein and peptide parenteral delivery[18].

1.3.2.1 Microparticles and Nanoparticles as drug delivery systems

Polymeric microparticles (which include microcapsules and microspheres) (1–1000 μm) were designed as depot formulations for long-term protein drug release (weeks to months). Compared to other formulations, microparticles provide enhanced flexibility in both fabrication methods and release rate[25,26].

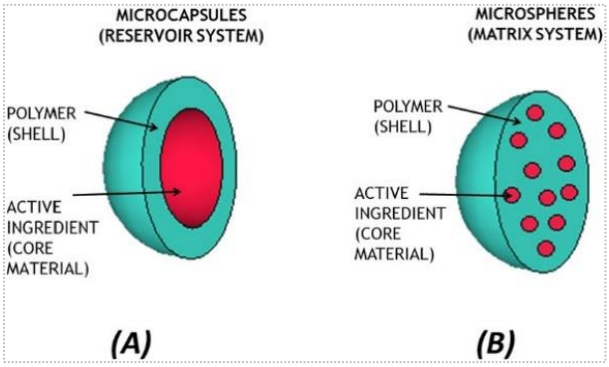

Figure 1-3. Differences between microcapsules and microspheres[27].

Common materials for protein or peptide drug preparations of microparticles include Chitosan, Dextran/Cyclodextrin, resistant starch/glycoprotein, resistant starch, β-cyclodextrin, Silica, Alginate, Hyaluronan acid, Chitosan, Gelatin, and poly (lactic-co-glycolic acid) (PLGA) [18,28]. One of the widely used materials for microparticles is poly (lactic-co-glycolic acid) (PLGA), as it is biocompatible, and biodegradable with favorable degradation rates. and has been approved by the FDA for clinical application[26,29]. PLGA microparticle formulations have nice long-acting performance for protein drug research

and delivery, and some marketed PLGA based microparticles for protein and peptide delivery formulation have been positively evaluated during clinical application, including Lupron Depot®, Decapeptyl®, Sandostatin LAR® Depot, and Somatuline® LA, and others[18].

Nanoparticles are delivery systems with particle sizes lower than 1 μm, which can be constituted by polymers, lipids, protein or inorganic materials. In tumor therapeutics research, the enhanced permeability and retention effect (EPR effect) is one of the theoretical cornerstones of nano medicine, and lots of nano delivery system were built to tackle this effect by adjusting materials, particle size, surface properties and responsible function. More “smart” functions were brought to the design and preparation of nanoparticles during last past years, for example, sensitivity towards pH, O2, enzymes, glucose levels, or other biological stimuli (Figure 1-4) in physiological environments[24]. Several “smart” nano delivery systems were designed according to those 'signalling' and 'receiving' models[30]. Although there were different opinions about the clinical application of the EPR effect [31], the development of biomedicine and nanotechnology will bring more solutions and tools for clinical diagnosis and treatment in the future [32].

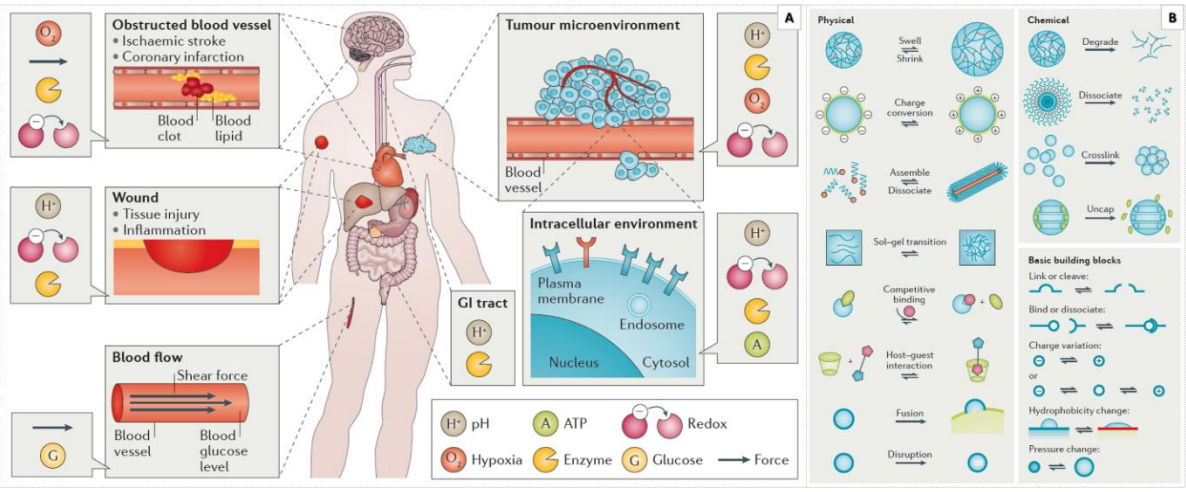

Figure 1-4, The strategy of bio-responsive formulation. (A)Typical physiological environments with associated biological stimuli, and (B) Typical bio-responsive actions of “smart” nanoparticles[24].

Those nano delivery systems exhibit good performance to increase the drug concentration in an organ or tissue that has suffered from the disease or tumor, and at the same time the nano delivery systems were also designed to decrease the side effect to the body during therapy. Also diagnostic functions were brought to some nano delivery systems named Theranostics (therapeutic-diagnosis co-work) during clinical

operation. Because of the rapid development of immunotherapeutics, lots of protein drug loaded nano delivery systems have been built, which include for example the immune checkpoint inhibitors delivery formulation.

During the research work of Gu et al., an in situ formed immunotherapeutic bio-responsive nano gel was developed, which encapsulated the anti-CD47 antibody to enhance the phagocytosis of local cancer cells by macrophages after surgery, and at the same time the local in situ formed gel can reduce side effects[33]. In the research of Philippe Maincent et al., PLGA nanoparticles were developed and characterized as a sustained release system for salmon calcitonin (sCT)[34]. Conjugation of antibodies to the surface of nanoparticles was also done during several research works, for example Sarmento et al, conjugated Fab fragments to PLGA nanoparticles[35], and Chudasama et al., presented a type of delivery system with covalently conjugated Fab fragments on PLGA–PEG nanoparticles, which improved availability, reproducibility and uniformity to enhance both biological activity and ease of manufacture[36]. Further, inorganic nanoparticles were also investigated for the protein drug formulation with special chemical capabilities of several materials, like Gold, Silicon[37], Graphene Oxide[38], or carbon nanotubes[39].

Some non-invasive administration approaches have also been improved during the last several years, which include the oral[40], nasal, pulmonary[41-44] and transdermal delivery[45,46] by nanotechnology.

1.3.2.2 Lipid-based formulations

Lipids are a class of organic compounds that exhibit a high extent of hydrocarbon-chains or their derivatives and are insoluble in water but soluble in organic solvents. They include natural oils, waxes, and steroids[47]. Lipids are basic parts of living systems in nature, and their functions include storage of energy, signaling, and acting as structural components of cell membranes. Lipid-based delivery systems include liposomes, solid lipid nanoparticles, oily suspensions, submicron lipid emulsions, lipid implants, lipid microbubbles, inverse lipid micelles, cochlear liposomes, and lipid microtubules, and lipid micro-cylinders (Figure 1-5)[48].

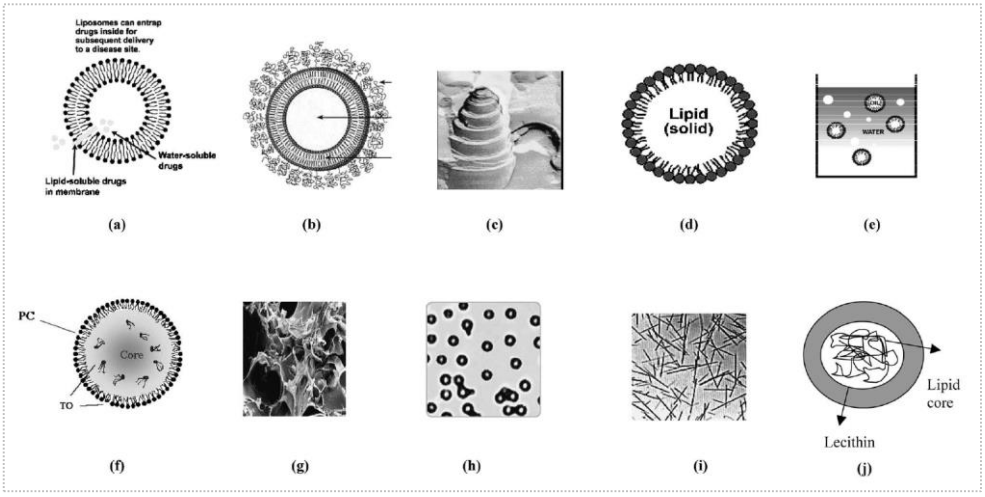

Figure 1-5, Different types of Lipid based carriers (a) Liposomes, (b) Stealth Liposomes, (c) Cochlear liposomes, (d) Solid lipid nanoparticles (SLN), (e) Oily suspensions(f) Lipid emulsions, (g) Lipid implants, (h) Microbubbles, (i) Microtubules, (j) Lipospheres [48].

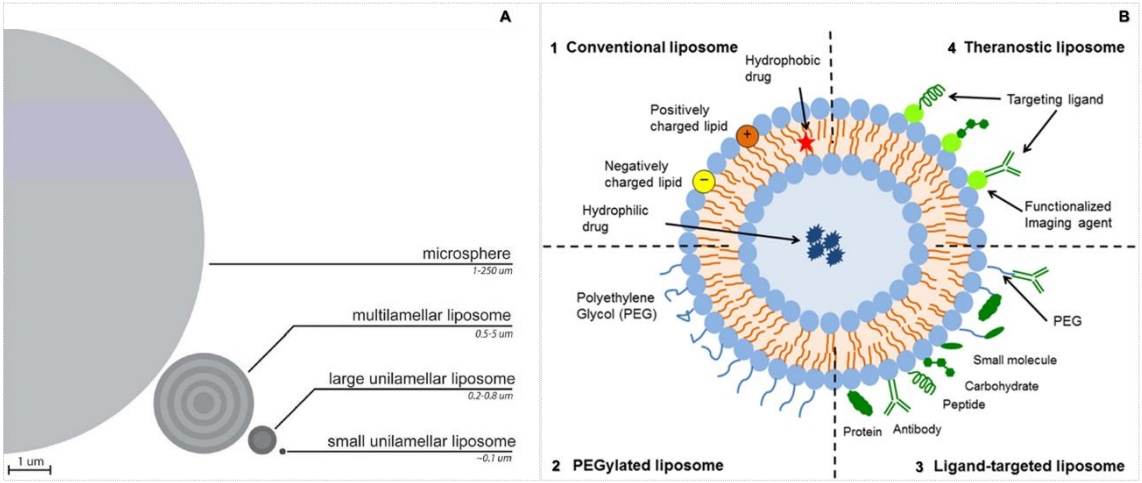

Figure 1-6 Liposome structures and modifications. (A) Size comparison of nano and micro particulate delivery systems; (B) Schematic representation of the different types of liposomal drug delivery systems[49,51].

Liposomes are microscopic phospholipid spherical bubbles with a lipid bilayer membrane structure, and encapsulated aqueous phase in the center and between the lipid bilayers[49,50]. Liposomes have high loading capacity, protection of loaded agents, good biocompatibility and biodegradability *in vivo*. There are different types of liposomes: Multilamellar liposomes vesicles (MLV) range in size from 500 to 5,000 nm and consist of several concentric bilayers. The large unilamellar vesicles (LUV) range in size from 200 to 800 nm and the small unilamellar vesicles (SUV) range around 100 nm in size and are formed by a single bilayer (Figure1-6, A). Through materials adjusting and preparation methods, liposomes with different properties such as size, surface charge, membrane flexibility and the agent loading mode for different clinical applications have been obtained (Figure 1-6, B)[49,50].

	Clinical Products (Approval Year)	Administration	Active Agent	Lipid/Lipid:Drug Molar Ratio	Indication	Company
1	**Doxil® (1995)**	i.v.	Doxorubicin	HSPC:Cholesterol:PEG 2000-DSPE (56:39:5 molar ratio)	Ovarian, breast cancer, Kaposi's sarcoma	Sequus Pharmaceuticals
2	**DaunoXome® (1996)**	i.v.	Daunorubicin	DSPC and Cholesterol (2:1 molar ratio)	AIDS-related Kaposi's sarcoma	NeXstar Pharmaceuticals
3	**DepoCyt® (1999)**	Spinal	Cytarabine/Ara-C	DOPC, DPPG, Cholesterol and Triolein	Neoplastic meningitis	SkyPharma Inc.
4	**Myocet® (2000)**	i.v.	Doxorubicin	EPC:Cholesterol (55:45 molar ratio)	Combination therapy with cyclophosphamide in metastatic breast cancer	Elan Pharmaceuticals
5	**Mepact® (2004)**	i.v.	Mifamurtide	DOPS:POPC (3:7 molar ratio)	High-grade, resectable, non-metastatic osteosarcoma	Takeda Pharmaceutical Limited
6	**Marqibo® (2012)**	i.v.	Vincristine	SM:Cholesterol (60:40 molar ratio)	Acute lymphoblastic leukaemia	Talon Therapeutics, Inc.
7	**Onivyde™ (2015)**	i.v.	Irinotecan	DSPC:MPEG-2000:DSPE (3:2:0.015 molar ratio)	Combination therapy with fluorouracil and leucovorin in metastatic adenocarcinoma of the pancreas	Merrimack Pharmaceuticals Inc.
8	**Abelcet® (1995)**	i.v.	Amphotericin B	DMPC:DMPG (7:3 molar ratio)	Invasive severe fungal infections	Sigma-Tau Pharmaceuticals
9	**Ambisome® (1997)**	i.v.	Amphotericin B	HSPC:DSPG:Cholesterol:Amphotericin B (2:0.8:1:0.4 molar ratio)	Presumed fungal infections	Astellas Pharma
10	**Amphotec® (1996)**	i.v.	Amphotericin B	Cholesteryl sulphate:Amphotericin B (1:1 molar ratio)	Severe fungal infections	Ben Venue Laboratories Inc.
11	**Visudyne® (2000)**	i.v.	Verteporphin	Verteporphin:DMPC and EPG (1:8 molar ratio)	Choroidal neovascularisation	Novartis
12	**DepoDur™ (2004)**	Epidural	Morphine sulfate	DOPC, DPPG, Cholesterol and Triolein	Pain management	SkyPharma Inc.
13	**Exparel® (2011)**	i.v.	Bupivacaine	DEPC, DPPG, Cholesterol and Tricaprylin	Pain management	Pacira Pharmaceuticals, Inc.
14	**Epaxal® (1993)**	i.m.	Inactivated hepatitis A virus (strain RGSB)	DOPC:DOPE (75:25 molar ratio)	Hepatitis A	Crucell, Berna Biotech
15	**Inflexal® V (1997)**	i.m.	Inactivated hemaglutinine of Influenza virus strains A and B	DOPC:DOPE (75:25 molar ratio)	Influenza	Crucell, Berna Biotech

* i.v. (intravenous); i.m. (intramuscular); HSPC (hydrogenated soy phosphatidylcholine); PEG (polyethylene glycol); DSPE (distearoyl-sn-glycero-phosphoethanolamine); DSPC (distearoylphosphatidylcholine); DOPC (dioleoylphosphatidylcholine); DPPG (dipalmitoylphosphatidylglycerol); EPC (egg phosphatidylcholine); DOPS (dioleoylphosphatidylserine); POPC (palmitoyloleoylphosphatidylcholine); SM (sphingomyelin); MPEG (methoxy polyethylene glycol); DMPC (dimyristoyl phosphatidylcholine); DMPG (dimyristoyl phosphatidylglycerol); DSPG (distearoylphosphatidylglycerol); DEPC (dierucoylphosphatidylcholine); DOPE (dioleoly-sn-glycero-phophoethanolamine).

Table 1-2. Clinically used liposome-based products[58].

Since the 1960s, the liposomes have been used in drug delivery research. The first generation liposome formulation (DOXIL®, a PEGylated liposome-based cancer therapeutic) went to clinical application in 1995, and more formulations were approved by FDA, which include the subsequent approval of DaunoXome (1996), DepoCyt (1996), Marqibo (2012), and the recently approved Onivyde (2015)[52]. Protein and peptide encapsulation in liposomes was developed for proteins like β-Glucuronidase, interleukin-2 and bevacizumab to prolong the acting time and decrease side effects[53-55]. Immunoliposomes are an elegant approach to use the target function of an antibody to enhance delivery of an active encapsulated drug to the respective tissue or organ (Table 1-2) [56,57].

1.3.2.3 Lipid based depot delivery systems for protein drugs

In addition, there are several other lipid vehicles that serve as long-acting formulations, for example the DepoFoam^{TM} particles, which were developed by Ye et al.[29]. The DepoFoam particles (MVL) are composed of non-concentric multiple lipid layers [59] (Figure 1-7). This non-concentric nature of the arrangement of lipid layers confers an increased level of stability and longer duration of drug release as a bolus injection. The MVL system was used for the protein and peptide drug delivery (IFN-α and IFN-α-PEG_{5000}) in form of a long-acting (sustained-release) platform, and it also showed nice storage stability performance (6 months) [60]. The MVL is also a good long-acting delivery system for antibodies. For examples, Sun et al. designed and developed the Bevacizumab-loaded multivesicular liposomes (Bev-MVLs) to increase the intravitreal retention time of bevacizumab and reduce the number of injection times. According the *in vitro* release test, the Bev-MVLs exhibited sustained release for more than 11 days, and during the *in vivo* treatment, the Bev-MVLs could effectively inhibit the thickness of choroidal neovascularization (CNV) lesion compared to a Bevacizumab solution at 28 days after treatment[55].

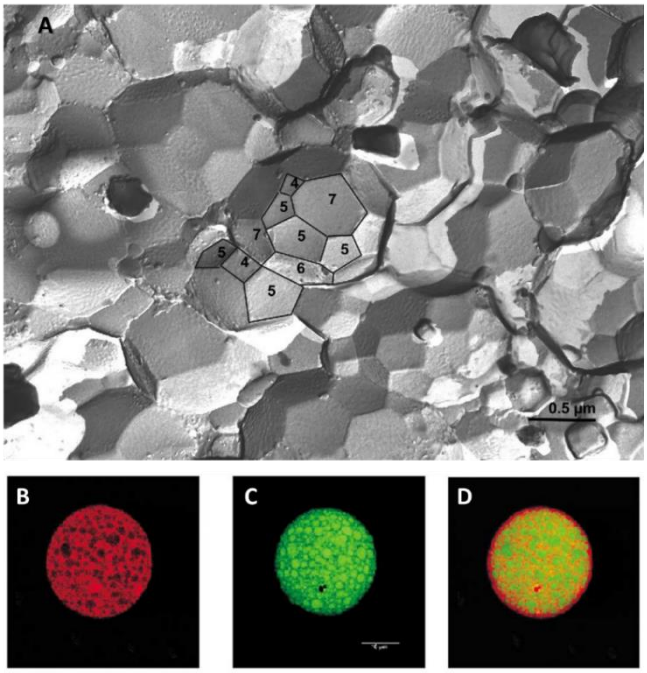

Figure 1-7, The MVL particle of DepoFoam Technology. (A) Freeze fracture electron micrograph of a MVL liposome showing random close packing of the internal chambers; The Pic B, C, D were taken by Confocal micrographs of DepoFoamTM particles recorded with (B) a red fluorescent dye (Rhodamine DHPE) labelling the lipids, (C) a green fluorescent dye (Bodipy disulfonate) labeling the aqueous phase. (D) In the merged image, the distribution of both the lipids and the encapsulated aqueous phases is seen. (The bar indicates 10 mm.)[59]

Implant systems are long-acting delivery systems that should be administrated into the body by a minor surgical procedure. The first approved biodegradable implant for

protein delivery was Zoladex®, which consists of PLGA and releases the drug over 3 months[61].

Solid Lipid Implants (SLIs) showed good long term release (60 days) performance and were prepared by screw extrusion during the work of Schulze et al [62]. Those SLIs also showed excellent properties with good biodegradability and biocompatibility *in vivo* in the work of Sax et al.[63]. Based on previous developments, Even et al [64], developed vaccine loaded SLIs, where the release from the SLIs was completed in 14 days. After that, Vollrath et al, brought the monoclonal antibody (Ranibizumab) into SLIs systems, and an sustained protein delivery over 18 weeks was achieved for age related macular degeneration (AMD) treatment[65,66].

Phospholipid-based Phase Separation Gels (PPSG) are a type of lipid formulation containing large amount of phospholipids (up to 70%), which was developed by the Zhang group[67,68]. The PPSGs were prepared using the ethanol dissolved phospholipids by stirring. When exposed to aqueous solution, where the lipids are insoluble, the formulation became the drug depot. Several peptides, which include Octreotide acetate and Leuprorelin acetate have been encapsulated in PPSGs by Gong and Fu et al [69,70], and the sustained release systems performed well both *in vitro* and *in vivo*, especially during Fu's work, where the therapeutic effect was maintained for up to 35 days[69]. Because some local irritations were caused by residual organic solvents contained in PPSGs *in vivo*, medium chain triglycerides (MCT) were added to increase the biocompatibility[68].

1.4 Vesicular Phospholipid Gels (VPGs) as delivery system

Vesicular Phospholipid Gels (VPGs) have a lipid concentration lower than PPSGs but higher than liposomes. They show long-acting release performance and good biocompatibility *in vivo*. The VPGs were originally described by Brandl et al. in 1994, and were prepared by high-pressure homogenization of highly concentrated phospholipid dispersions[71,72]. VPGs are semisolid depot gels formed by phospholipid vesicles, which were first described as highly concentrated lipid dispersions, which normally consist of 300 ~ 600mg/g lipids. VPGs have a gel-like consistency formed by numerous densely packed vesicles[73]. VPGs are different from hydrogel liposome-systems, often called “liposome-gels”, where a hydrophilic polymer like poly-acrylate forms a three-dimensional matrix and liposomes are embedded in this hydrogel-matrix. VPGs may be formed by phospholipids both in the gel and the fluid state[29,71,74]. In the last decades, they have emerged both as storage-stable intermediates for vesicular liposomes yielding extraordinarily high encapsulation efficiencies as well as semisolid depot formulations for sustained release of drugs[75,76]. The aqueous compartments are both within the cores and in-between the vesicles, so the system is suitable for entrapping hydrophilic compounds including proteins. During early investigations by Brandl et al., VPGs with phospholipid concentrations ranging from 300~500 mg/g (lipid/VPG) were prepared and the release rate of the model drug (Calcein) was decreased by increasing lipid concentrations (Figure 1-8)[74].

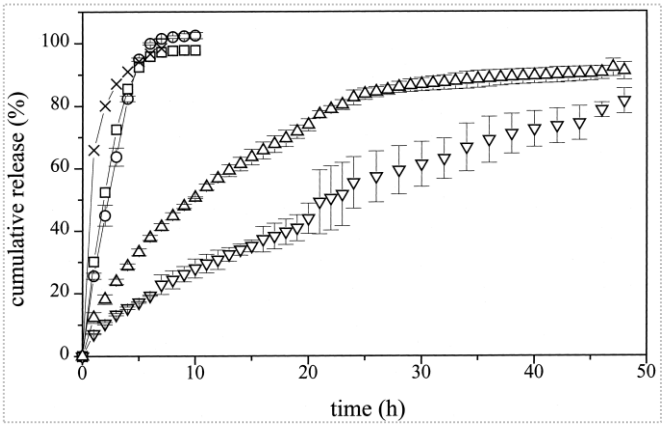

Figure 1-8, Calcein release from gels with different EPC contents. Cumulative release versus time: - X -, 300 mg/g; - □ -, 350 mg/g (single runs). - ○ -, 400 mg/g; -△ -,450 mg/g; - ▽ -, 500 mg/g (mean ± S.D.; n=3 each)[74].

The microstructure of VPGs was investigated by Freeze fracture transmission electron microscopy (FF-TEM) in the research of Brandl et al., and their internal structures show an aqueous core encapsulated in lipid, which is very similar to MVL microstructure (Figure 1-9). The particles after VPG redispersion were also measured by negative stain electron microscopy (NS-EM)[71] .

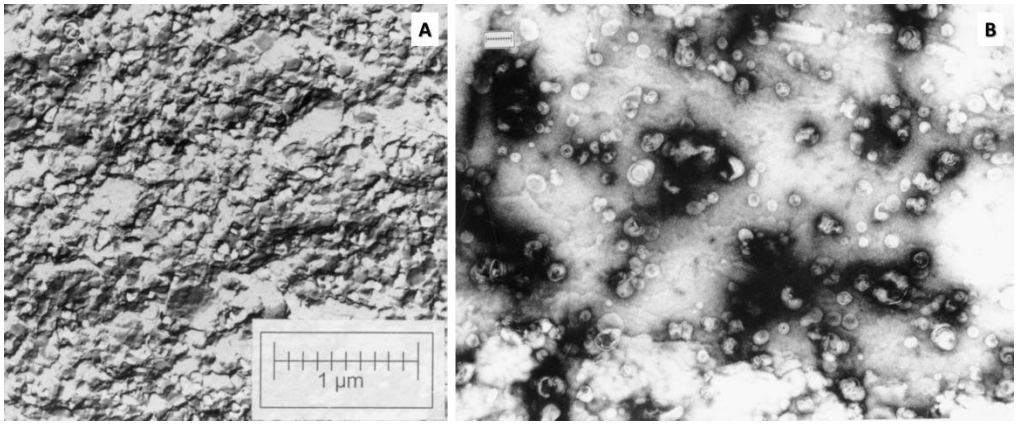

Figure 1-9. The microstructure of VPGs by electron micrograph, (A) FF-TEM micrograph of the 40% EPC-gel (VPG); (B) NS-EM micrograph of the non-autoclaved gel after redispersion (the scale bar is 100 nm)[71].

1.5 Preparation methods of Vesicular Phospholipid Gels (VPGs)

1.5.1 High-pressure homogenization (HPH)

The first preparation method for VPGs was high-pressure homogenization (HPH) described in the patent of Brandl et al. in 1994[72]. The first VPG preparation method was to mix the dry powdered lipids with aqueous medium, and then feed those coarse dispersions into a lab-scale high-pressure homogenizer (Gaulin Micron Lab 40, APV Homogenizer, Lübeck, Germany). The homogenization conditions were 70 MPa and 10 homogenization cycles.

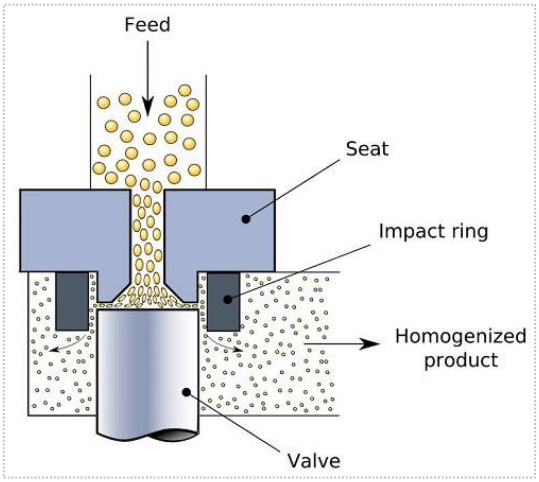

Figure 1-10, The basic mechanism of high pressure homogenization[77].

Because of strong shearing force and heat stress during HPH, this method was only suitable for small molecule drugs, but not for protein drugs. Using the HPH preparation method, several small molecule drugs were encapsulated by VPG, which included Calcein[74], carboxyfluorescein[71], vincristine[78], 5-Fluorouracil[79], and Gemcitabine[80].

1.5.2 Dual asymmetric centrifugation (DAC)

The Dual asymmetric centrifugation (DAC) is a type of “Speed Mix” equipment: while the conventional centrifugation constantly pushes the sample material outwards, the sample container vial is turned around itself center (vertical axis), which pushes the sample material constantly towards the center by additional rotation forces[81]. This method supports a soft and even shearing force to mix samples, and is especially suitable for high viscosity samples like solid/semisolid powders or gels. Compared to with the HPH, the DAC shows no bubble formation, is easy to operate and to clean, and

delivers highly reproducible results (Figure 1-11). For different formulations, different preparation parameters or conditions were optimized, which included the type of mixing containers, mixing beads, mixing time and rotation speed.

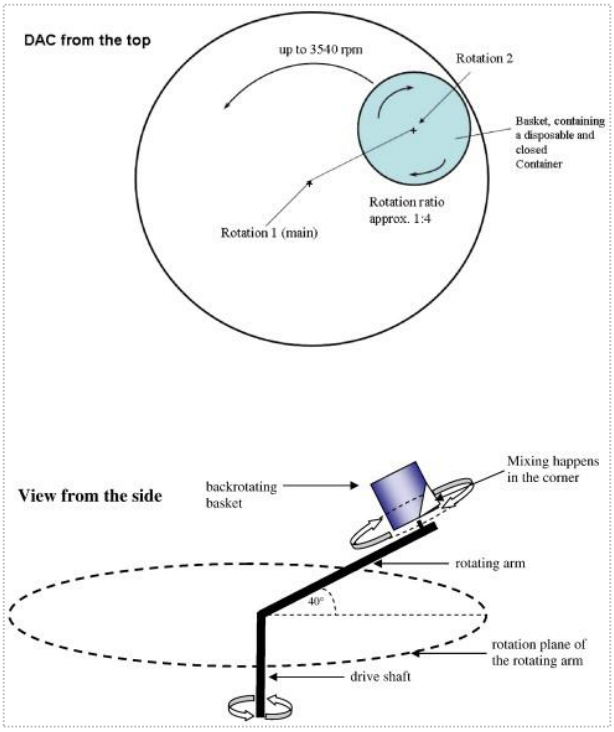

Figure 1-11, Schematic drawings of the principle of dual asymmetric centrifuging[81].

The DAC method was first developed by Massing et al. in 2007. In the work of Tian et al. a therapeutic protein (Erythropoietin, EPO) was incorporated into VPG formulations with the DAC method (2009), and the VPG showed good performance as a depot formulation for sustained protein release. In the following research, a monoclonal antibody (mAb), granulocyte-colony stimulating factor (G-CSF) and interferon-β-1b (IFN-β-1b), were encapsulated into VPGs by DAC, to achieve long-acting release formulations[82-84]. The DAC method also showed good performance for small molecules. For example, Qi et al. encapsulated the Ara-C and obtained a feasible extended release profile. Balsevich et al, used the SAFit2 loaded VPG formulation during *in vivo* treatment for stress-related psychiatric disorders (major depression), and the SAFit2 loaded VPG formulation could support a chronic treatment for at least 30 days[85].

Currently, the DAC is the standard method for VPG formulation preparation, but this method still has weaknesses: Because the protein drug loaded VPG is a very high viscosity formulation, the necessary mixture time for large volume preparations should be very long to achieve a homogeneous drug distribution, but the long preparation time will increase the sample temperature, which may jeopardize protein stability.

1.5.3 Magnetic Stirring (MS)

The Magnetic Stirring (MS) is a very common mixing method for pharmaceutical formulations, and it has very strong versatility for different situations. The MS is the necessary pretreatment for VPG preparation by HPH, which will deliver the coarse dispersion of lipid/buffer systems [74]. The shearing force of MS is weak, so it is not possible to crush all solid particles in the dispersion mixture during the short time preparation. Some VPGs were prepared by MS methods to obtain depot delivery systems. In 2013, the Zhang group reported a low phospholipid concentration (300mg/g) VPG for 5-hydroxy-fluorescein-thymopentin (5-FAM-TP5) delivery, which was prepared by the MS. In 2015 Exenatide was encapsulated in VPGs (20%, 30% and 40%, w/w%) by MS. During the measurement of 5-FAM-TP5 loaded VPGs and Exenatide loaded VPGs, the sustained release capabilities were demonstrated well *in vivo*, which means MS is a useful method to prepare low lipid concentration VPG. Breitsamer et al., also test the MS method to prepare VPG formulation during their research[86,87]. Since the shearing force from Magnetic Stirring is not strong enough, is it not easy to mix the lipids and buffer very even during short time; but the versatility and scale-up capability during pharmaceutics research is strong.

1.5.4 Extrusion (EX)

The Screw Extrusion is a type of widely used technology in plastic, food and pharmaceutical industry, which can mix using special shaped screws (Figure1-12) [88]. During previous pharmaceutical research, the lipid and drugs were mixed and extruded for implants preparation. In the work of Even et. al. and Vollrath et al., the twin-screw (TSC) extruder was used during the protein loaded lipid implant formulation preparation; then Breitsamer et al[85,87]. used this method for VPG preparations, and different lipid concentration VPGs had been prepared and tested, to achieve good quality VPGs. The advantages of EX are with the good control of the shearing force to make the sample more homogenous, and at the same time, it also has strong continuous production capability for scale-up production manufactory.

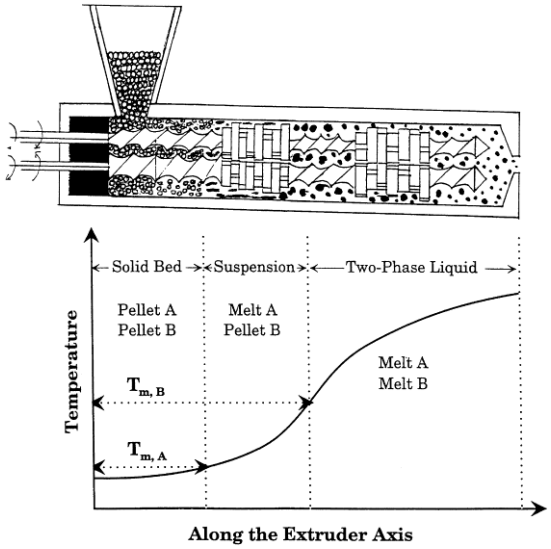

Figure 1-12, Schematic diagram of a twin-screw extruder, in which a pair of immiscible polymers are extruded under a preset temperature profile along the extruder axis[88].

1.6 Purification and analysis of protein loaded VPGs

In contrast to small molecules, the structural and physical stability are core parameters during protein formulation research and development. VPGs are formulations with extremely high concentration of phospholipids, and because the protein should be pure to avoid the lipid interference during the analysis, the purification of protein from the VPG is important step.

1.6.1 The organic solvent extraction of VPGs

During previous work, the protein loaded VPG formulations were extracted using an organic solution directly, which is a similar workflow as for small molecular drugs. Tian et al. used chloroform to extract the EPO and mAb out of the VPG, and DMF was used for G-CSF purification. After extraction, SDS-PAGE, RP-HPLC and other methods could be used for protein analysis; Neuhofer et al. systematically screened different organic solutions for extraction of IFN-β-1b (table 1-3)[84], Breitsamer et al. developed an extraction method for the mAb by RP-HPLC measurement, where the mAb loaded VPG was diluted with pure ethanol (1:1) and mixed evenly[87]. After centrifugation at 8000 g for 10min the clear supernatant protein solution was used for injection into RP-HPLC and subsequent protein quantification.

solvent	Lipoid E80	IFN-β-1b	placebo VPG	IFN-VPGs
acetonitrile 100 %	not soluble	soluble	not soluble	not soluble
acetonitrile/methanol (50/50)	soluble	soluble	soluble	not soluble
methanol 100 %	soluble	soluble	soluble	not soluble
chloroform	soluble	soluble	not soluble	not soluble
tetrahydrofuran	soluble	soluble	soluble	not soluble
ethanol	soluble	soluble	soluble	not soluble

Table 1-3 Solubility of 5 % (m/m) Lipoid E80, 20 % (m/m) placebo VPGs and IFN loaded VPGs in different solvents determined by visual inspection[84].

The disadvantages of the extraction method using organic solvents lies in changes of the tertiary structure of the protein, and ultimately protein denaturation. Because of that, the organic solvents extraction methods are not the first choice for biomolecule drugs activity analysis, and new methods should be developed for protein loaded VPGs.

1.6.2 Chromatographic methods for protein purification

Protein purification is an important operation during the biological and biopharmaceutical research, and after purification, the pure protein sample can be used further. Methods for protein purification include the chromatographic and electrophoretic methods, and chromatography includes the Gel filtration (SEC), Ion exchange (IEX), Affinity and Fast protein liquid chromatography (FPLC), while the electrophoretic methods include the preparative electrophoresis and Isoelectric focusing (IEF). Because of the high number of samples generated in protein loaded VPGs research, the multi-channel capability, low cost and high efficiency performance of the chromatographic methods were necessary. The basic mechanism of different chromatography methods is presented in Figure 1-13.

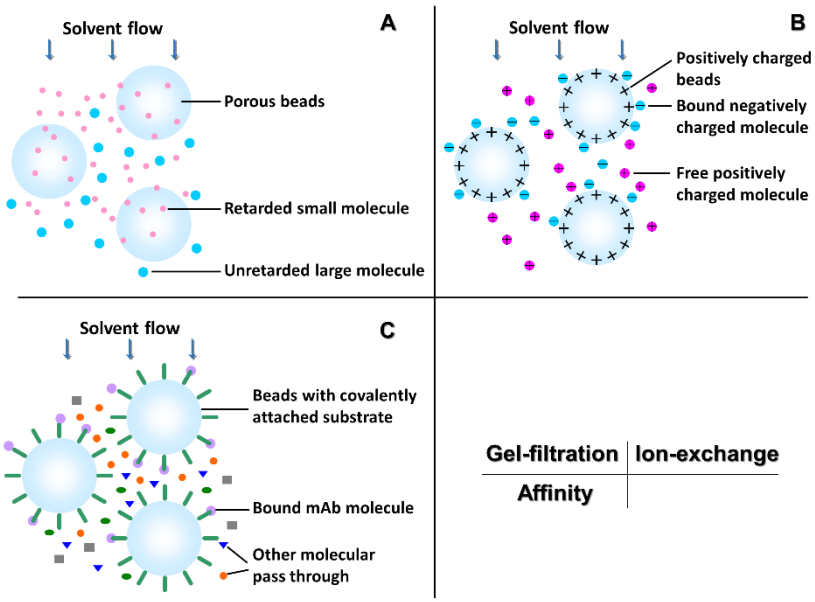

Figure1-13, The separation of macromolecules on chromatography column methods, (A) Gel-filtration chromatography; (B) Ion-exchange chromatography; (C) Affinity chromatography (Copyright © motifolio.com)[89].

The gel filtration can separate different sized macromolecules by different bead pore sizes. The first gel filtration method for protein purification was reported by Lindner et.al. in 1959 [90], and was used for antibody purification by Mizejewski et. al. in 1979 [91]. The columns can be packed with different material beads, like dextran polymers (Sephadex), agarose (Sepharose), or polyacrylamide (Sephacryl or Bio-Gel P). Zienkiewicz et al. published a protein purification protocol for lipid-rich plant tissues using a Sephadex G25 column and a feasible solubilization buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 40 mM DTT, 0.5 % (v/v) carrier ampholytes pH 3–10, and 0.002 % (w/v)

bromophenol blue). After the extraction and purification, the protein samples were analyzed by electrophoresis.

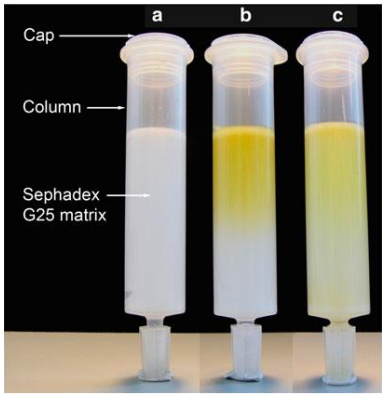

Figure 1-14, Filtration of protein extracts prepared from olive pollen through a column containing Sephadex-G25 medium (PD-10 column, GE Healthcare Biosciences AB). (a) PD-10 column after equilibration and before sample loading. (b & c) The same column as above after sample filtration (b) and protein elution (c), respectively[92].

Because most proteins have a net surface electrostatic charge at all pH-values except for the pH = pI (isoelectric point), the ion exchange (IEX) protein purification can separate proteins based on differences in surface charges and their corresponding electrostatic interaction with the column beads[93]. IEX-chromatography can be classified into three types depending on the elution mode, which includes elution by salt gradient, elution by pH-gradient, and elution by affinity. IEX-chromatography also can be separated into anion exchange and cation exchange. The early investigations for IEX protein separation methods were reported by Partridge et.al. in 1949[94]. In 1953, Isliker et. al. reported the antibody purification method by IEX[95], Knudsen et al. reported the antibody process-scale purification[96].

The separation methods based on affinity are powerful tools for protein purification, which can include the use of selective antigens, anti-antibodies, bacterial Fc receptors, Protein-A-mimetic ligands, metals-, thiophilic interactions, lectin- and affinity tags like his-tags[97]. During the preparation of a recombinant protein, the purification with selective tags is a very common method, like the His-tagging method based on the affinity for metal due to a histidine-rich sequence, or Protein A-methods, where protein A binds the heavy chain within the Fc region of most IgG proteins strongly. In 1970, Cuatrecasas et. al. described protein and peptide purification methods by affinity chromatography[98], and in 1976 Skvaril et. al. used the commercially protein A sepharose for IgG purification[99]. The affinity methods deliver high purity and yield, which are easily and reliably achieved.

1.6.3 Lipid removal methods for high lipid concentration samples

For the high lipid formulation samples, the common methods are not enough to purify the protein from samples, and some removal materials should be added for the protein purification. Since the 1970s, these methods have been used in food industry, and were established for biology samples to remove lipids and endotoxin [100-102]. They can be applied to remove lipids either in the sample preparation step, the solid phase extraction step or the sample purification step, with minimal adverse effect on non-lipid compounds[102]. During the application, these materials include, Florisil®, Lipid Removal Agent (LRA), Micro Cel-E® and Calflo E®. The lipid removal agent (LRA) is a large surface area material, where the synthetic adsorbent is crystalline calcium silicate hydrate, and the detailed chemical composition is as follow (Table 1-4).

Na_2O	MgO	Al_2O_3	SiO_2	P_2O_5	SO_3	K_2O	CaO	TiO_2	Fe_2O_3
0.26	0.8	2.82	56.78	0.23	0.21	0.33	37.38	0.13	0.93

Table 1-4, Major Chemical Composition of LRA Adsorbent (%) as analyzed by the X-ray fluorescence (XRF) method[101].

Gordon et. al. used the LRA to clean the lipid from human plasma high density lipoprotein during the proteomic study[103]; and Sakurai et al. also used the LRA to treat the plasma samples before the lipoprotein proteomic analysis[104]. Since the LRA remove the lipids from samples by physical adsorption, the interaction with protein structure is weak, therefore the risk for structural perturbations is rather low.

1.7 Erosion and Release test models for semi-solid lipid formulations (VPG)

1.7.1 Flow-Through Cell (FTC) model

Since VPGs are semi-solid formulations, the release testing is different compared to liquid or solid formulations, and the *in vitro* release testing (IVRT) needs special test models[105]. The first *in vitro* erosion and release testing experiment model for VPG depot systems was developed by Tardi et al. in 1998, and is called the Flow-through cell (FTC)[74].

That model was designed with the donor chamber in the center of release cells, and a channel of rectangular cross section of 5*5 mm in order to ensure a constant contact area of 250 mm^2 between donor and acceptor compartment which should change not very much during release, even if the upper layers of the preparation disappear via erosion. The acceptor chamber is also a 50 mm long channel but with a semicircular cross section of 2.5 mm radius and in a volume of the acceptor phase of 500 ml. The semicircular cross-section was found to facilitate the laminar flow of the acceptor medium, and the release buffer at a special flow rate controlled by a pump. A schematic drawing of the cell is given in Figure. 1-15.

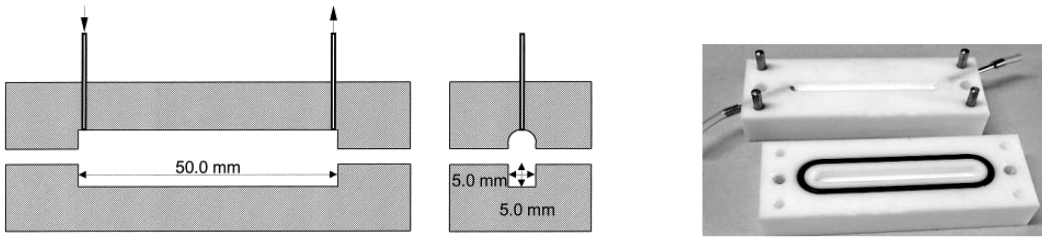

Figure 1-15, Schematic drawing of the release cell. On the left, the longitudinal section and cross section is shown. On the right the actual instrument is shown[71,73].

This model is feasible to investigate the release behavior of semi-solid gel formulations like VPGs *in vitro*, and it has been shown to work fine during research for VPG formulations[73,79]. But, this release test model still has some problems that need to be solved. First, in the real tissue, the VPGs would not have contact to the flowing liquid (blood in vessel) directly, they rather should penetrate the tissue and vessel wall. Also, the VPG surface can be shredded and parts of this drug-containing gel will be dragged by the solvent, without releasing the drug. That means the drug and lipid release from the VPG depot is different compared to Tardi's model. Second, in Tardi's release model high amounts of about 1 g VPGs are necessary for testing. But during protein drug

delivery research, small volume test system is necessary because of limited valuable sample volume during early investigations[74]. Last, in Tardi's release model, the only release test markers were the small molecular drugs, but there was not enough information about the release statues of drug, which could be released as free molecules or released while still encapsulated in liposomes[19,71,74].

1.7.2 The Dialysis model for VPG investigation

The release testing using dialysis bags is a common method for the drug release measurement from lipid vehicles[106-110] (Figure 1-16). For VPGs and similar formulations were investigated by the Zhang group, the dialysis bags were also used during the *in vitro* release measurements [111], and in their experiments, the molecule weight cut-off (MWCO) of the dialysis bags is 8000–14 000 Da for exenatide (Mw: 4000 Da) loaded VPGs, and exenatide was released from VPG as free molecule. A major advantage of the dialysis method is the fast and easy operation. In the release mechanism study for multivesicular (MVL) liposomes by Manna et al [110], the different MWCO dialysis membranes were also used, and the different release states were investigated. These studies show the different release behavior for different release phases.

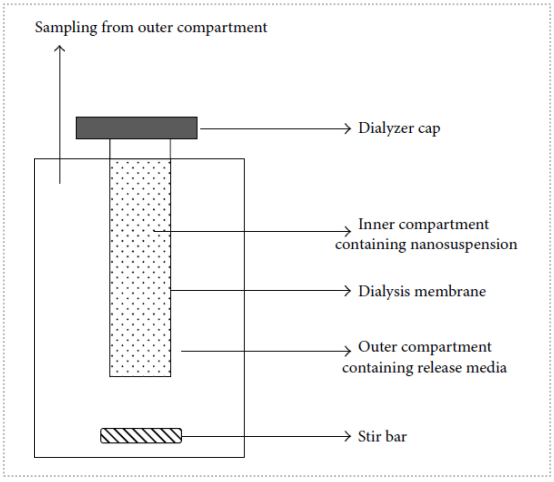

Figure 1-16, The Schematic of the release test method using a dialysis bag[107].

In the dialysis model, the formulation will release into the surrounding release buffer however the VPG is injected subcutaneously, which means the correlation to *in vivo* release and penetration is limited.

1.7.3 The Agarose-Gel (AGG) release test model

The agarose gels were also used in the depot formulation release studies. The pore size of the agarose gel can be controlled by adjusting the agarose concentration of the gels[112,113]. In the research of Thi et al. implant release was investigated with the agarose gel model[114], and Jensen et al. used the UV-imaging measurement for the insulin loaded implant release study where the release rate can be analyzed directly online (Figure 1-17)[115]. The cylinder samples could also be cut out of the agarose gel for the quantitative analysis

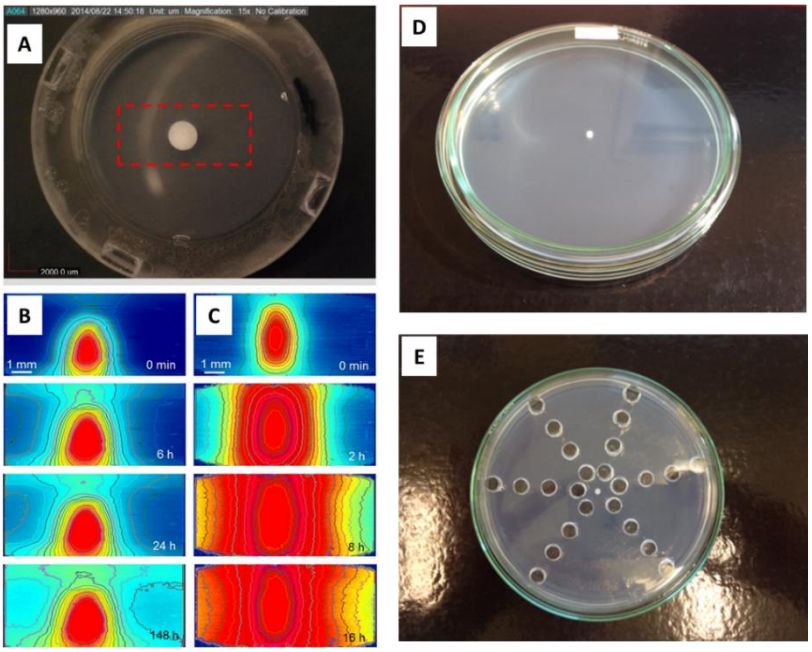

Figure 1-17, the agarose release model during the implant release behavior analysis. (A) A photograph (top view) of a human insulin containing lipid implant placed in the cylindrical quartz cell (15 mm (id) × 5 mm). UV absorbance maps of human insulin release from Sterotex implants with (B) 10% (w/w) and (C) 20% drug load in 0.5% (w/v) agarose hydrogel; (D) Pictures of the destructive gel-sampling set-up with lipid implants of human insulin placed in a petri dish (9 cm (id) x 5.5 mm in height) filled with 0.5% (w/v) agarose gel, pH 7.40, prior to sampling (E) and after completion of the experiment; samples had been withdrawn at a specific distance from the implant at different time points[115].

The agarose gel release model can support the release into surroundings that mimic tissues, which could come close to the subcutaneous administration of VPGs.

1.7.4 Simple and easy release models

Peschka et al. developed a simple *in vitro* release model in glass vials, to study the release kinetics of liposome encapsulated material. In that model the liposomes were mixed with 1% agarose gel as the bottom layer, and the upper layers were covered with 2% agarose gel[116].

In the research of Even et al, the Eppendorf tubes release model was used during the release investigation of ovalbumin loaded lipid implants as sustained release test

model[117]. Breitsamer et. al. used this method for a VPG release study, and the EPO loaded VPGs were tested for the short-term release comparison after different shearing force treatments[118].

1.7.5 Development of new release test models for VPG erosion and release testing

The important evaluation standards for long-acting formulations are pharmacokinetic performance and bioavailability *in vivo*. Hence, new *in vitro* models are required, to provide miniaturized, easy to operate and tissue-mimicking release test systems that will be more suitable for the VPGs research. The *in vitro* release test system should predict the pharmacokinetics and bioavailability performance *in vivo*. In 1997, the FDA defined the *in vitro* – *in vivo* correlation (IVIVC) for extended release (ER) (oral dosage) forms as "a predictive mathematical model describing the relationship between an *in vitro* property of a dosage form and a relevant *in vivo* response"[119,120] (Figure 1-18).

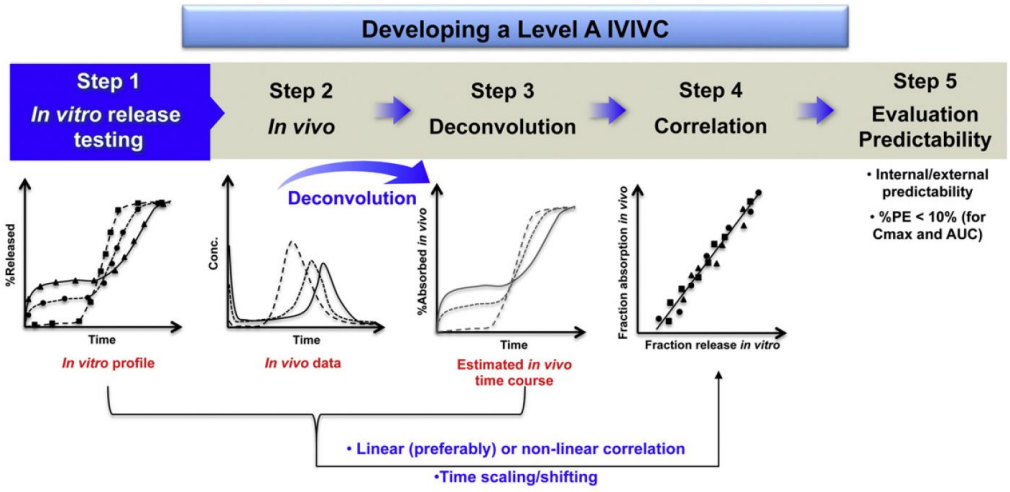

Figure 1-18 Procedures of developing a Level A IVIVC, and Step 4 shows a one-to-one linear correlation [119].

The VPGs are semi-solid formulations with very high phospholipid concentration, used as depot formulations for subcutaneous(SC) administration, in particular for peptide and protein drugs. VPGs consist of densely packed liposomal compartments and release drug via erosion and diffusion. Different *in vitro* models were used to determine release rates for VPG formulations in the past, but their quality was disputable due to a lack of *in vivo / in vitro* correlation. In fact, the assigned release behavior for certain formulations was depending on the used release model.

1.7.6 The erosion and released mechanisms study of semi-solid lipid formulations

Since the research of Tardi et al, the erosion and release mechanisms have been investigated with the flow through cell models, and according to their research, the erosion was rate limiting for the overall release. The release behavior of different VPG groups were different: The low lipid concentration VPGs (300mg/g) almost spontaneous disintegrated, the middle lipid concentration VPGs (350 – 400 mg/g) showed zero-order erosion kinetics for 4–6 h, and the high lipid concentration VPGs (450 – 500 mg/g) followed square root of time kinetics for 13 or 21 h, which is typical for matrix-controlled diffusion[74]. The differences in microstructures of the different VPGs were investigated by Brandl et. al., and the respective microstructures where responsible for the different release behavior.

In the research of Manna et al, the release mechanisms of bupivacaine loaded multivesiculas liposomes (BPV-MVLs) were investigated by Cryogenic-scanning electron microscopy (Cryo-SEM) and Confocal laser scanning microscopy (CLSM), and the results were correlated to the release behavior measurements. Decreasing particle sizes and loss of internal “polyhedral” structure of the MVLs over the duration of release were detected, indicating erosion and rearrangement of the lipid layers, which leads to an increased release rate (Figure 1-19)[110].

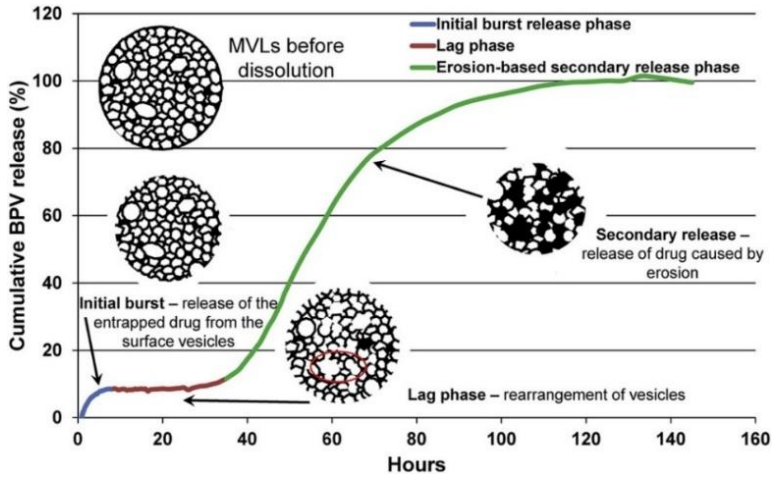

Figure 1-19. Schematic detailed possible release mechanism of BPV from the MVLs[110].

Comparison of different *in vitro* release testing (IVRT) experiments of semi-solid dosage forms is not easy, because , experimental parameters like dissolution medium, membrane, temperature, and speed also have an influence on the mechanism and kinetics of the release [105].

1.8 Long-term storage studies of protein loaded VPG systems

The storage capability is an important part of formulation development before clinical application. The VPGs are semi-solid lipid gels, and the gel's structure is not stable enough during long term storage, because the lipid and liquid phase can separate. At the same time, the stability is also the core parameter of protein loaded formulations. The key parameters include: the chemical stability of lipids and proteins, the protein drug bio-activity, the release performance and others.

1.8.1 Storage stability studies

Several groups of VPGs samples are prepared for our stability experiments, and the formulations will be investigated after preparation, and after 1 month and 3 months of storage. The stability samples will be separated into small individual packages and be stored at 4°C and 25°C (room temperature) environment and nitrogen atmosphere. The stability indicating methods include: SDS-PAGE analysis, texture analysis and rheology measurements, the release experiments and DLS analysis. High performance thin layer chromatography (HPTLC) is also performed for the lipid stability analysis[121].

Based on preliminary stability study results, the preparation method for protein drug loaded VPGs will be adjusted. We aim to build a long-term stable protein delivery system for the clinical application in the future.

1.8.2 Freeze-drying and storage

Freeze-drying (Lyophilization) is a well-established drying process for stabilization of biological molecules[122]. The drying is very gentle and may not affect to lipid and protein stability negatively, that means the preparation of protein loaded VPGs for long term storage should be possible[123]. The LyoRx sensor can be used to measure the electrical resistance and temperature of the product, which can determine the freezing point of sample [124,125], and it were used for condition control. For protein loaded VPG formulations the freeze-drying will be investigated.

1.8.3 Frozen storage

The Frozen state storage is an easy method to store samples, and most chemical reactions will decrease their speed at these low temperatures. Therefore, this can also be a possible method for long term storage of the protein loaded VPGs, if protein and

lipid integrity is not negatively affected by freezing and thawing. The frozen treatment and storage would support a short path for the protein loaded VPG formulations storage with low technical condition.

1.9 Aim of the thesis

There are four main purposes in this thesis.

The first purpose is to develop a new *in vitro* test model for protein loaded VPG formulations and to investigate their erosion and release behavior. For these studies, different *in vitro* release models will be tested and compared with the *in vivo* PK results, to allow the selection of an appropriate model for further using. With the new release models, we try to set up the *in vivo - in vitro* correlation.

The second purpose is to develop a new protein purification and analysis method for protein (mAb) loaded VPGs. Because the normal extraction methods cannot remove high lipid concentrations from formulations and preserve the protein activity at the same time, we want to develop a better method to purify the antibody. The final protein activity and lipid concentration are the core parameters, and different methods will be tested. The proteins structural stability and protein affinity should be measured to prove this method works well.

The third purpose is to develop new methods for protein (mAb) loaded VPGs long-term storage. The differently stored formulations will be extracted, analyzed and evaluated by new release test model from previous research. With a feasible long-storage method, the VPG formulations would be step forward towards clinical application.

The last purpose is the scale-up manufacture of VPGs depot formulations. There will be different behavior between the small volume preparation and the scale-up manufacture of the VPGs with different methods. The capability of scale-up manufacture by different methods will be compared and evaluated.

These four purpose is about the protein loaded VPG systems, to investigate the release situations from VPGs, to build better and easier methods for protein purification and analysis, and to develop the scale-up manufacture method. This work will help to explain release mechanisms of VPGs, and to push the VPG depot formulation a step ahead to clinical applications in future.

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Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Phospholipid

The main component of the VPGs is Egg phosphatidylcholine 80 (EPC 80), which was supplied from Lipoid GmbH (Ludwigshafen, Germany). The EPC 80 contained at least 80% phosphatidylcholine (PC) and other components such as phosphatidylethanolamine (7.0-9.5%), lysophosphatidylcholine (<3%), sphingomyelin (2-3%) and triglycerides (<3%).

2.1.2 Fluorescein isothiocyanate-dextran (FITC-dextran, 70 kDa)

The FITC-dextran (with an average molecular weight of 70 kDa) was obtained from SIGMA (Product number: 90718-1G). It was used as a macromolecular drug model during the formulation research. FITC-Dextran is a hydrophilic powder with good stability in aqueous solution.

2.1.3 Monoclonal antibody (IgG)

The monoclonal antibody (mAb) LMU-1 human IgG (145 kDa) was used in these studies as active Pharmaceutical Ingredient (API). The protein concentration of stock was 120 mg/mL determined by UV-spectroscopy.

2.1.4 Lipid removal agent (LRA)

The LRA (Product number 13360-U, Supelco) is a patented synthetic mineral that has a unique affinity for lipids, lipoproteins, and endotoxins. LRA is a synthetic calcium silicate hydrate fine powder. It is composed of 30% calcium oxide, 48% silicon dioxide, and residual levels of sodium, magnesium, and iron.

2.1.5 SAFit2

SAFit2 (CAS No. 1643125-33-0, MW. 802.99) is a hydrophobic small molecule, which has the strong capability as a novel FKBP51 antagonist during chronic treatment. The SAFit2 powder was obtained from the Max-Planck-Institute for Psychiatry (Munich, Germany).

2.1.6 Chemical reagents

NaCl and Na2HPO4·2H2O were obtained from Bernd Kraft. KCl was obtained from Applichem. KH2PO4, NaN_3, (CHAPS (3-[(3-Cholamidopropyl)-dimethylammonio]-propane-sulfonate) and 1, 4- Dithiothreitol (DTT) were obtained from Merck KGaA.

Acetonitrile (>99.9%, HPLC gradient grade, A/0627/17) was purchased from Fisher Chemical, Chloroform was obtained from Merck KGaA, Trifluoroacetic acid (TFA) and Sucrose were purchased from Sigma-Aldrich (Steinheim, Germany), Polyvinylpyrrolidone (PVP) Kollidon® 17 PF was produced by BASF.

Albumin, Bovine (BSA), (for biotechnology, 0332-500G, was obtained from VWR Chemicals).

Highly purified water was produced by a Purelab Plus, (USF Elga, Germany).

2.1.7 Membranes for the release test model development

Different pore size membranes were used in these studies, which include a 1.0 µm pore size membrane (Regenerated cellulose, CAT No. 10410012, Whatman™), a 5.0 µm pore size membrane (Cellulose Acetate, 12342-47-K, Sartorius Stedim), a 41.0 µm pore size membrane (Nylon Net, NY4104700, Merck Millipore), and an 80 µm pore size membrane (nylon Net, NY8004700, Merck Millipore).

2.2 Methods

2.2.1 VPG preparation methods

2.2.1.1 High-pressure homogenization (HPH)

Egg phosphatidylcholine 80 (EPC 80) was mixed with phosphate buffer (20 mM, pH 7.4) for 2 h under magnetic stirring. Then, about 25 g raw dispersions were homogenized at 700 bar for 10 cycles using a high-pressure homogenizer Micron Lab 40 as described earlier as ‘one-step' liposome preparation [1].

2.2.1.2 Dual Asymmetric Centrifugation (DAC)

Phospholipids and buffer were brought into the 25 mL sample containers (Polypropylen, white, 15-25ml cylindrical container), and treated with 3500 rpm, 45 min, and at 2~8°C

by DAC (Speedmixer TM DAC 150 FVZ, Hauschild & Co. KG, Hamm, Germany) [2], and because the DAC container size, the samples volumes were set between 1 to 5 g.

2.2.1.3 Magnetic Stirring (MS)

Triangular Stir Bars (L 40 mm, bar diam. 14 mm, 442-0389, VWR) were used to mix the EPC 80 powder and phosphate buffer together for 2 hours under Magnetic stirring in the 250 mL glass Beaker. During the preparation, the rotation rate set in 100 rpm in the first 30 min, and then turned up to 300 rpm. During the long-term storage study, the sample volume was 3 g (or mL), and during the scale up manufacture study, the sample volume was 40 g (or mL).

2.2.1.4 Extrusion (Ex)

The Extruder preparation method was following the method of Breitsamer et al[3,4]. The EPC 80 powder and phosphate buffer were mixed 5 min before extrusion by magnetic stirring. The working parameters of the ZE-5 Mini-Extruder (Three-Tec GmbH, Seon, Switzerland) were set at 300 rpm, 30 °C, and the diameter of the terminal nozzle is 0.4 mm. The product of extrusion was collected in micro-centrifugation tubes (1.5mL), and then stored at 2-8 °C before use.

2.2.2 Drug loaded VPG preparation

2.2.2.1 FITC-Dextran (70 KDa) loaded VPG preparation

The FITC-Dextran was dissolved in 20 mM PBS to obtain a 20 mg/mL stock solution. 375μL of the FITC-Dextran stock solution was added into a DAC container, and then a series of different lipid weights was added (450 mg for 30% lipid VPG, 600 mg for 40% lipid VPG, and 750 mg for 50% VPG), and combined with the corresponding volume 20 mM PBS (675 μL for 30% lipid VPG, 525 μL for 40% lipid VPG, and 375 μL for 50% VPG). Then all samples were prepared by DAC (45 min, 3500rpm, 2~8 °C), and after preparation, all VPGs were stored at 2-8 °C until further use.

2.2.2.2 The preparation of mAb loaded VPGs

The stock solution of mAb (120 mg/mL) was concentrated to 200 mg/mL by ultrafiltration (VIVASPIN 20, 50 K MWCO PES) with centrifugation (8000g 10min 3 times, and then 10000g 10min 3 times). Placebo VPGs (3.5 g), 30%, 40% and 50% lipid content in gel (w/w), were prepared as control group, and the PBS (20mM, pH7.4) was added to the gels as buffer in preparation. A series of mAb loaded VPGs (3.5 g) were prepared: their

phospholipid concentrations were set to 30%, 40% and 50% (w/w) respectively, with included 1.05 g, 2.1 g, and 1.75 g EPC80, and 2.45 mL, 2.1 mL, and 1.75 mL solution (buffer and protein solution), and the antibody concentrations were 1%, 5% and 10% (w/w). All samples were prepared by dual asymmetric centrifugation system (DAC) with a speed of 3500 rpm for 45 min at 2~8 °C.

2.2.2.3 SAFit2 loaded VPG preparation

SAFit2 is a hydrophobic small molecule under research for the treatment of stress-related psychiatric disorders (major depression), and was encapsulated into VPGs for an *in vivo* therapeutic experiment in animals. The SAFit2 powder was dissolved in ethanol to obtain a 20mg/mL stock solution. 1 g EPC80 was dissolved in 2 mL Ethanol in a DAC container, and 1 mL SAFit2 stock solution was added into the solution under gentle mixing. The ethanol was then removed by evaporation for 3 days (25°C, 10mbar, in Memmert Vacuum Drying Cabinet with CVC 2000 Vacuum Pump) during the early phase of reducing the pressure, there were multiple steps of waiting in case of boiling, (at 300mbar for 2 hours until samples were stable, and then at 100mbar for 2 hours until samples were stable). The lipid-drug mixture was then hydrated with 1 mL 10 mM PBS (pH7.4), and the VPGs were prepared by DAC (45 min, 3500rpm, 2~8°C). The VPGs were filled into syringes and closed with a rubber stopper, and stored at 2-8 °C before use.

2.2.3 The antibody loaded VPG extraction method

20 mg of the antibody loaded VPG (mAb@VPG) was placed into a 2 mL micro-centrifugation tube, then mixed with 930 µL 20mM PBS buffer using the Capmix™ for 2 second and afterwards 50 µL 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) water solution (4% w/v) and 60 mg (LRA) powder were added (see a detailed discription in Chapter 3).

Capmix™ was used to mix each of the samples three times for 1 sec. at a time. A 1 mg/mL mAb solution was used as positive control, the negative control consisted of 20 mM PBS. All samples were incubated for one hour at 37 °C and a shaking rate of 90 rpm. Afterwards all tubes were centrifuged at 4 °C, 10000 g, for 10 min. After centrifugation, the supernatant (about 600 µL) was removed by pipetting and the extracted mAb solutions were stored in 1.5 mL tubes for the following measurements.

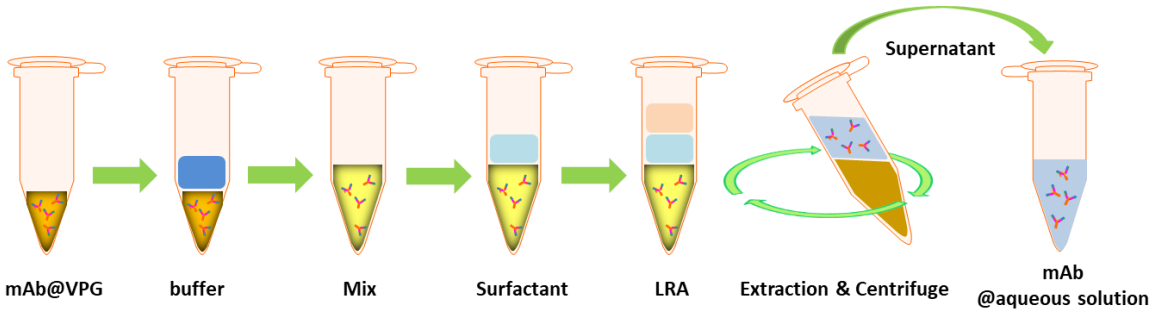

Figure 2-1, Scheme of operation steps for mAb extraction from VPG.

2.2.4 The measurements of physical VPG parameters

2.2.4.1 Rheology and Texture Analyzer for VPGs

The viscosity is an important parameter of VPG formulations, and was measured using a rotational Rheometer (Physica MCR 100, Anton Paar GmbH, Ostfildern, Germany) with plate-and-plate design (PP-25). During the measurement, the temperature was set to 25 °C, the shear rate was set to 10-100 s^{-1}, and 1g of VPG was used for measurement. The viscosity of the VPGs was compared at the fixed shear rate of 37.9 s^{-1} in the increasing rotation rate step.

The gel strength was measured by Texture Analyzer (TA.XT Plus, Stable Micro Systems, UK). For each measurement about 1.5 g of VPG was carefully filled in a 2 ml centrifugation tube. The measurement probe with a 4 mm flat tip was pushed into the gel with a speed of 0.5 mm/sec to a final penetration depth of 4 mm from the top surface. The gel strength was determined as the maximum force point, about 8 seconds after start.

2.2.4.2 Thermogravimetric Analysis (TGA)

To investigate the difference in the moisture content of different VPG formulations, the TGA (TGA 2950 HI-RES, TA Instruments, New Castle， USA) was used in the research. For each formulation, about 5~10 mg gel was brought into the microbalance, and the sample was heated from 25 to 250°C using a temperature ramp of 10 °C/min under nitrogen atmosphere, and each formulation was measured in triplicates (n=3).

2.2.4.3 VPG surface morphology microscopic analysis

2.2.4.3.1 Confocal 3D Laser Scanning-Microscope

Approximately 10 mg of the VPG formulations, were taken for the surface microscope analysis, and the surface 3D topography was investigated by Confocal 3D Laser Scanning-Microscope (VK-X250, KEYENCE) (operated by Felix Berger, KEYENCE).

2.2.4.3.2 Scanning Electronic Microscopy (SEM)

The morphology of VPG surfaces was analyzed by SEM (FEI Helios G3 UC), with EDX-Detector, Raster Transmission Detector and Focused Ion Beam (FIB). Approximately 10 mg of the VPG formulation were attached on carbon self-adhesive tape on aluminum stubs, and investigated at a voltage of 2 kV at the magnification of 200X, 500X, 1000X, 5000X, and 10000X.

For investigation of the released fraction of VPGs, about 5 µL released sample solutions were dropped on the smooth surface of Regenerated cellulose filter paper (GE, Whatman™, CAT No. 10410012), and incubated for 30 min, before SEM analysis.

2.2.5 Release models for VPGs

2.2.5.1 Flow-Through Cell model (FTC)

This method is one of the most used measurement methods for semi-solid formulations. In this work, a small FTC was produced of Teflon, with a 25 mm length donor chamber, and a rectangular cross-section of the donor chamber of 5×5 mm. There are two tubing adapters at the two endings of the donor chamber (Figure 2-2 A). The 500mg sample in the donor chamber can release into the flow through buffer directly. Tubing adapters of the cells were connected with a 60mL syringes (50mL (60mL) Luer Lock, 8300006682, SOFT-JECT®) by tubing. The syringes supplied the release buffer at a special flow rate controlled by a syringe pump (LA-160, Landgraf Laborsysteme HLL GmbH). The release fractions were analyzed after the respective release period.

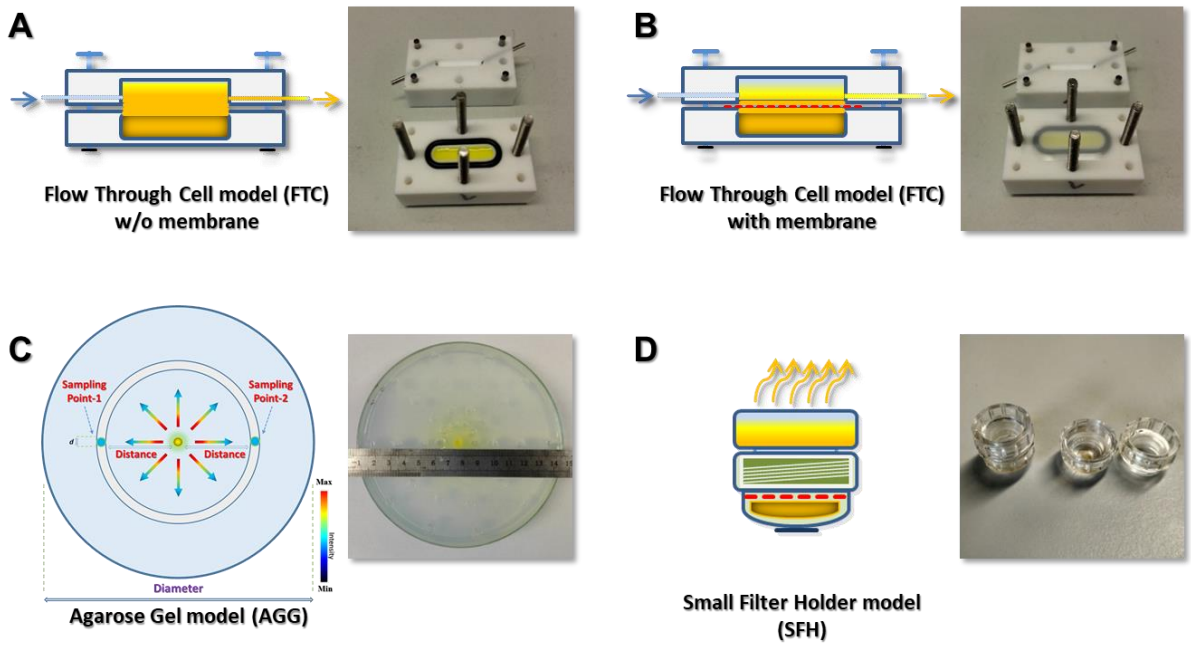

Figure 2-2: Schematic illustration of release models for VPGs. (A) The Flow Through Cell model (FTC) without (w/o) membrane. (B) The Flow Through Cell model (FTC) with membrane. (C) Agarose Gel model (AGG). (D) Small Filter Holder model (SFH).

2.2.5.2 Flow-Through Cell model (FTC) with membrane

We added a membrane (Cellulose Acetate membranes with 5 µm pore size) into the FTC to adjust the release behavior of the semi-solid formulation. This membrane is used to simulate the tissue, and therefore, the release behavior of the VPGs is expected to be more similar to *in vivo* conditions (Figure 2-2 B). Other parameters were set as for the previous FTC model. The release samples were taken at different time points.

2.2.5.3 Agarose Gel Model (AGG)

The AGG model consisted of 0.5 % (w/v) agarose gel, hereof about 350 mL was placed into 15 cm diameter Petri dishes. On the other hand, 10 cm diameter Petri dishes were also used during the SAFit2 loaded VPG release study, and the agarose gel volume was decreased to suitable volume. For every formulation, the concentrations of 10mg/g drug/VPG (FITC-Dextran 70 KDa or SAFit2) were kept the same. A circular hole was made in the middle of the agarose gel by a plastic pipe (made by Omnifix®-F syringe, cut the adapter terminal), and the diameter of hole was about 6.3 mm, the depth was about 10 to 12 mm. Considering the thickness of agarose gel, the maximum amount of sample that could be placed in the hole was 340 mg (formulations) or µL, and all test groups were used and analyzed in triplicates (n=3). The VPG samples were placed into

the sample hole in the middle of the agarose gel (Figure 2-2 C), then the circular hole was closed by adding a drop of agarose gel. The dishes were put into the temperature incubation shaker (60 rpm, & 37 °C), and all dishes were sealed. Each Petri dish was covered with aluminium foil to protect the samples from light.

Then, the cylindrical samples were taken by the same type of plastic pipe. (The pipe was made by syringe, Qmnifix®-F, BRAUN, and the cylindrical diameter was about 6.3mm, the cylindrical length was the same as the depth of agarose gel.) The cylindrical samples were taken from specified positions (at 1, 2, 4 and 6 cm from the center point) as reference [5], and from the diagonal projection positions two cylindrical samples were mixed together for enough samples volume (Figure 2-2 C). After the gel sampling, the gel samples were pushed through a by 25 mm syringe filter to remove the gellant and to receive a clear solution (0.2 µm pore size cellulose acetate membrane), and the samples solutions were analyzed. Also, the real-time release of this model was measured by a fluorescence/UV imaging system (e.g. ChemiDoc™ Imaging system), and the imaging parameters were set as follows: Application-Blot is Alexa488, the imaging sight size is 22.2 x 16.7cm, and the exposure time was set to 0.001, 0.002 and 0.003 sec. The colored picture was used for the qualitative comparison of the release rate. The original pictures were then uncolored, and based on the mean signal intensity values the quantitative release (penetration) was calculated.

2.2.5.4 Small Filter Holder model (SFH)

The Small Filter Holder model was derived from a small membrane filter holder (re-usable syringe filter holders, for 13 mm membrane filters, 16514, Sartorius Stedim Biotech, Germany), by closing the Luer adaptor part of the hold, and opening a big window in the exit tubing side (Figure 2-2 D). For the release study, the 300 mg VPG samples were injected into the chamber and covered with a nylon membrane (40 µm pore size). These SFH with release samples were placed into a 50mL centrifuge tube, which was filled with release buffer. Then all these tubes were put into a horizontal shaker (60 rpm, 37 °C). The release was tested 1, 2, 3, 7, 13, 16, and 21 days, after preparation. The SAFit2 & phospholipids release was quantified by RP-HPLC and Phospholipid test assay kit respectively.

2.2.6 Analysis of the release fractions

2.2.6.1 Phospholipid concentration measurement during the release study

The phospholipid concentration was measured by Colorimetric-enzymatic method [6], which with the Phospholipid test assay kit (LabAssay™ Phospholipid 1300 Tests, 296-63801), and the general assay principle was placed in Figure 2-3. UV absorptions were measured in 96 well plates with the FLUOstar® Omega microplate reader at excitation wavelengths of 600 nm and 700 nm.

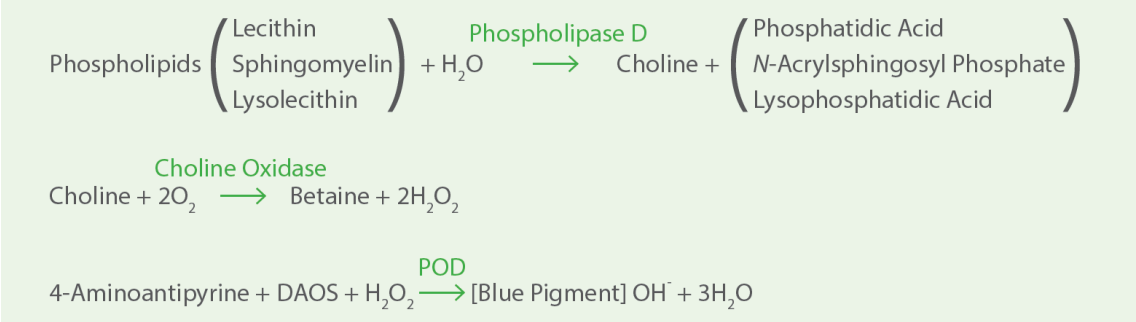

Figure 2-3 The assay principle of LabAssay™ Phospholipid test assay kit [6,7].

2.2.6.2 Fluorescence Spectrophotometry for FITC-Dextran release

FITC-Dextran (70K Da) was quantified by Fluorescence Spectrophotometry, which offered a high sensitivity down to the nanogram per milliliter range. The FITC-Dextran was directly quantified after dilution with PBS. The FITC-Dextran powder was diluted with PBS (20 mM, pH 7.4) to 50.0 µg/mL stock solution, and then 2/3 dilution to 0.578 µg/mL (take 600 µL high concentration solution mix with 300 µL buffer), the standard curve was used for quantitative analysis (R^2 = 0.9845). The excitation wavelength was set to 492 nm and the emission wavelength set to 518 nm, and the black 96 well plates (Nunc™, 96 wells black, MicroWell™) were used in the measurement, and 250 µL of each sample were used per well in the plate reader model analysis.

2.2.6.3 mAb quantification by RP-HPLC

During the release study, the method for mAb quantitative analysis followed the method of Breitsamer [4]. The mAb in the released fraction solutions was diluted with pure ethanol (1:1) and mixed accurately. And then, the sample solutions were quantified by RP-HPLC using a Dionex UltiMate™ 3000 (Thermo Fisher Scientific, Waltham, USA) equipped with an auto sampler (UltiMateTM WPS-3000SL) and a fluorescence detector (UltiMateTM FLD 3400RS). Mobile phase A was composed of 10% (m/m) acetonitrile, 0.1% (m/m) trifluoroacetic acid (TFA) and highly purified water (HPW). Mobile phase B

was composed of 0.1% (m/m) TFA in acetonitrile. A Phenomenex Jupiter C4, 5 µm column (Phenomenex Inc. Torrance, USA) was used as a solid phase and the oven temperature was set to 50°C. The gradient was set to 0-100% B in 20 min. mAb was detected at a retention time of 15.2 minutes using fluorescence (λexc. = 280 nm; λem. = 350 nm). The method was qualified with samples containing defined concentrations of mAb, phospholipids, and mixtures after extraction. The mAb concentration in each sample was calculated using a calibration curve (R^2 = 0.9993).

2.2.6.4 Quantification of SAFit2 by RP-HPLC

The SAFit2 concentration in released fractions was quantified by RP-HPLC, and this part was done by Breitsamer et al. Samples were prepared by dilution with ethanol (1:2), subsequent mixing using a vortexer and centrifugation (10 000 rpm, 10 minutes at 4 °C). By this extraction method, an extraction yield of 100.2 ± 1.6% was achieved. A YMC-Triart C18 3 µm column (YMC Europe GmbH, Dinslaken, Germany) was used in combination with a UltiMate™ 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a UltiMate™ FLD 3100 fluorescence detector. The mobile phase A and phase B were prepared as described in chapter 2.2.6.3, and a multi-step gradient (0-15 min: 20-100% mobile phase B; 15-17 min: 100% mobile phase B; 17-18 min: 100-20% mobile phase B; 18-21 min: 20% mobile phase B) at a flow rate of 0.5 ml/min was used for quantification. SAFit2 was detected at λem = 310 nm (λex = 280nm) with a retention time of approximately 14 min. The method was qualified using samples with known concentrations of SAFit2, BSA, phospholipids or mixtures of them after using the extraction protocol described above. These measurements were performed by Breitsamer et al, and more detailed information was described in her thesis [4].

2.2.6.5 Particle analysis of the release fractions

2.2.6.5.1 Particle size analysis by Dynamic Light Scattering (DLS)

The particle size measurements were performed by dynamic light scattering using the Zetasizer nano ZS (Malvern Instruments, UK). Different amounts of VPGs were accurately weighed and dispersed in a buffer by Vortex. The VPG samples were diluted with 20mM PBS solution 100x to 1000000 X (volume to weight ratio, taking 1980 µL PBS to dilute the 20 mg VPG for the 100 times dilution). The obtained dispersion was further diluted (1:10) before measurement with the Zetasizer Nano ZS (Malvern Instruments, UK). For the VPG release experiments, the released fraction was also

measured. The Z-Average size, Intensity Mean size, PDI and Derived Count Rate (DCR) were taken for further analysis.

2.2.6.5.2 Particle size analysis by Nanoparticle Tracking Analysis (NTA)

The NTA LM20 (NanoSight Ltd.) was used for these experiments and it provided both a direct, real time view of nanoparticles and a comprehensive particle-by-particle size distribution analysis. The particles movement is recorded via light scattering by a CCD camera and the software tracks the particles as light-scattering centers moving under Brownian motion. Following parameters were used for the measurements: The wavelength was set to 405 nm, with 65 mW power, and the detection hold was set as 10 at maximum output and room temperature [8]. The sample was diluted with 20 mM PBS to suitable particle concentrations.

2.2.7 *in vivo* pharmacokinetic investigation of SAFit2 loaded VPG

2.2.7.1 The *in vivo* PK study of SAFit2 loaded VPG

The SAFit2 loaded VPG formulations (with 30% and 50% lipids) are tested *in vivo* after subcutaneous (SC) administration in mice, and the mice type is C57Bl/6N (n=7 in 30% lipids VPG group, and n=8 in 50% lipids VPG group). As a control SAFit2 in aqueous solution (20% EtOH, 40% propylenglykol, 5% Tween 80, 5% PEG 400 in 0,9% saline) was also tested by subcutaneous administration. All formulations were injected subcutaneously with a dose of 10 mg/kg body weight. Blood samples are taken at pre-determined time-points and analyzed by LC-MS/MS. Relative bioavailability was calculated with the following equation. These parts study was performed in accordance with the European Communities' Council Directive 2010/63/EU by the Max-Planck-Institute of Psychiatry, Munich, Germany.

2.2.7.2 The pharmacokinetic parameter calculation of SAFit2 loaded VPG

After *in vivo* experiments, the data was processed and parameters were calculated by Dr. Jilong Wang and Prof. XiaoJiao Du (Institutes for Life Sciences and School of Medicine, South China University of Technology, Guangzhou, China) using a non-compartmental model in the Data Analysis System 3.2.6 pharmaceutical *in vivo* calculation software (BioGuider co., Shanghai). According to these results, the *in vivo* PK results and *in vitro* release behaviors will be compared.

2.2.8 Protein stability measurements

2.2.8.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein stability was studied with the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which to investigate aggregation and degradation qualitatively. For these experiment, the 1.0mm*10 well 3-8 % Tris-Acetate Gel (NuPage™, Invitrogen, EA0375BOX), Tris-Acetate SDS Running Buffer (20X) (NuPage™, LA0041), Unstained Protein Standard, (HiMarkTM, LC5688) and LDS Sample Buffer (NuPage™, NP0007) were used. Biorad Powerpac 200 was used to run the gels for 60 min with a constant voltage of 150 V. Afterwards, the gels were stained with a silver staining kit (SilverXpress®, Invitrogen, LC6100). The 4–12% Bis-Tris Gel 1.0mm*12 well (NuPAGE™, Invitrogen, NP0322BOX) was used for the protein reducing SDS-PAGE analysis.

2.2.8.2 High-Performance Size Exclusion Chromatography (SE-HPLC)

The aggregation and degradation of an antibody would lead to changes in the molecular size, which can be tested by SEC-HPLC. Separation and analysis were performed on a Waters 2695 Separation module (Waters GmbH, Eschborn, Germany) connected to a Waters 2487 Dual l Absorbance Detector (Waters GmbH). A YMC Pack Diol 300 column was used, and the detection wavelengths were set to 280 nm and 214 nm. Before analysis, all sample solutions were centrifuged with 10000g (10min, 4°C) for big particle removing, and then 20 µL sample solutions were injected for each sample, using a mobile phase consisting of 100 mM sodium phosphate, 200 mM NaCl at pH 7.0. The flow rate was set to 0.5 mL/min.

2.2.8.3 Ion exchange-high-performance liquid chromatography (IEX-HPLC)

For the antibody variant separation, a pH Gradient was applied using a weak cation exchange column (054993, ProPac® WCX-10, Thermo). The mobile phase A consisted of 2.4 mM Trizma® Base, 1.5 mM imidazole and 11.6 mM piperazine at a pH of 6.0; mobile phase B only differed in the pH value (pH 9.5). The flow rate was set to 0.5 mL/min, and the multi-step gradient method of chromatography is given in Figure 2-4.

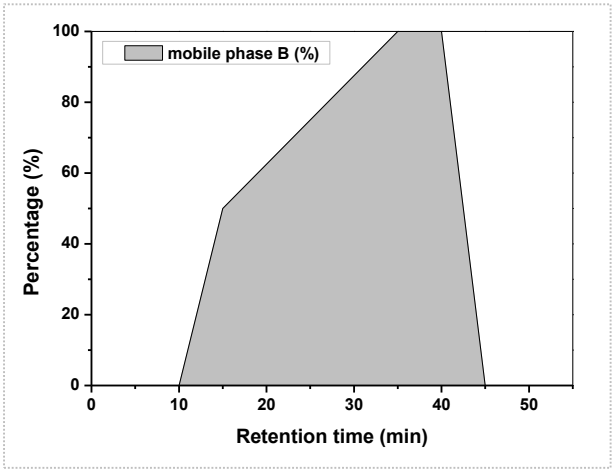

Figure 2-4 The multi-step gradient method of IEX-HPLC

Since the pH values after mAb extraction were very high and the samples peaks were shifted during the measurement, the samples had to be diluted with mobile phase A for further IEX-HPLC analysis. The pH values are shown in Table 2-1, and the 1:9 dilutions were used for the further IEX-HPLC measurements in the next step.

		Mixture Ratio																
		0:1	1:0	1:1	1:2	1:3	1:4	1:5	1:6	1:7	1:8	1:9	1:10	1:11	1:12	1:13	1:14	1:15
pH value after diluted by buffer	PBS_Extraction + IEX-mobile_phase-A	6.07	8.45	7.37	6.94	6.82	6.74	6.66	6.59	6.53	6.48	**6.44**	6.40	6.37	6.36	6.32	6.28	6.26
	mAb_Extraction + IEX-mobile_phase-A		8.47	7.45	6.94	6.83	6.71	6.64	6.56	6.45	6.40	**6.37**	6.33	6.32	6.30	6.27	6.26	6.24

Table 2-1 The pH values after extracted samples and IEX-mobile phase A

2.2.8.4 Circular Dichroism (CD) spectroscopy

Circular Dichroism (CD) spectroscopy is a useful method for the investigation of the secondary and tertiary structure of proteins. With the far UV (180- 250 nm) the secondary structure of proteins can be detected and the near UV (250-320) displays the tertiary structure. For the far UV measurement, a highly purified buffer solution is necessary, but the extraction method to gain these purified solutions must be gentle enough to avoid artefact formation. The spectra were recorded at 25 °C with the Jasco J-810 spectropolarimeter (JASCO Deutschland GmbH, Pfungstadt, Germany). Cuvettes with an optical path of 1.0 cm were used for near UV detection and the absorbance level of UV was set to 0.7~1.2 for best results. The buffer solution and VPG extraction solutions were measured as blanks and subsequently subtracted from each mAb loaded VPG group. For each sample, spectra were smoothed with 7 smoothing points and the mean residue ellipticity was calculated as described elsewhere.[9-12]

2.2.8.5 The bio-affinity analysis by Bio-Layer Interferometry

The affinity performance is a key capability of the antibody which is related to its bioactivity in a certain protein formulation. The BLI (bio-layer interferometry) technology is a sensitive and easy method to investigate the interaction between molecules, resulting in real-time data on protein interactions. During these measurements, the Fc and Fab regions were measured to test preservation of bio-affinity stability after extraction. The sensors selected were Protein-A Biosensors (18-5010) and Anti-Human Fab-CH1 2nd Generation (FAB2G) Biosensors (18-5125). The 20 mM PBS extraction solution was set as a reference and the standard curve ranged from 15.6 µg/mL to 1000 µg/mL. During the BLItz^{TM} BLI analysis, the pH value of the sample was a very important parameter: the extracted samples were diluted at a rate of 1:3 with 20 mM phosphate buffered saline (PBS) to gain a pH 7.4 (Table 2-2), to keep the sample solution in the same pH value during the measurement. By comparison to standard curves, the concentrations of Fc and Fab were calculated.

		Mixture Ratio					
		0 : 1	**1 : 0**	**1 : 1**	**1 : 2**	**1 : 3**	**1 : 4**
pH value after diluted by PBS	**PBS_Extraction + PBS** (pH 7.40)	7.40	8.45	7.60	7.54	**7.45**	7.45
	PBS_Extraction + HPWater	6.74	7.45	7.14	/	**/**	/

Table 2-2 The pH values situation after PBS-Extraction mixed with the PBS

The Qctet^{TM} BLI system was also used for the BLI analysis. The main parameters were set as following: Cycle times was set to 200 ms, average Count to 20, sensor offset to 3000, the Assay time to 60 s, at a flow rate of 400 RPM. By comparison to standard curves, the concentrations of Fc and Fab were calculated.

2.2.9 The Freeze-drying and Freezing of VPG formulations

2.2.9.1 The Freeze-drying methods of mAb loaded VPGs

VPGs and mAb loaded VPGs were prepared by DAC, then 3 g VPG (or drug loaded VPG) were filled into the freeze drying glass vial very carefully (Injection Bottles 10R, 45.0*24.0/1.00mm, MGlas AG), and a small magnetic stirring bar (Stirring Bars, cylindrical 20X6 mm, 442-4523, VWR) was added into every vial for reconstitution later, and the rubber stopper was placed on the top of vial with exhaust gaps. The CHRIST EPSILON 2-6D laboratory scale freeze-dryer was used, and the freeze-drying protocol is given in Figure 2-5.

Progress Phase		1	2	3	4	5	6	7	8
		Loaded	Freezing	Freezing	Primary drying	Primary drying	Primary drying	Secondary drying	Secondary drying
Time	h		1:10	1:30	0:30	0:30	30:00	3:00	9:00
Temperature	°C	20	-50	-50	-50	-20	-20	4	4
Pressure	mBar				0.100	0.100	0.100	0.100	0.100

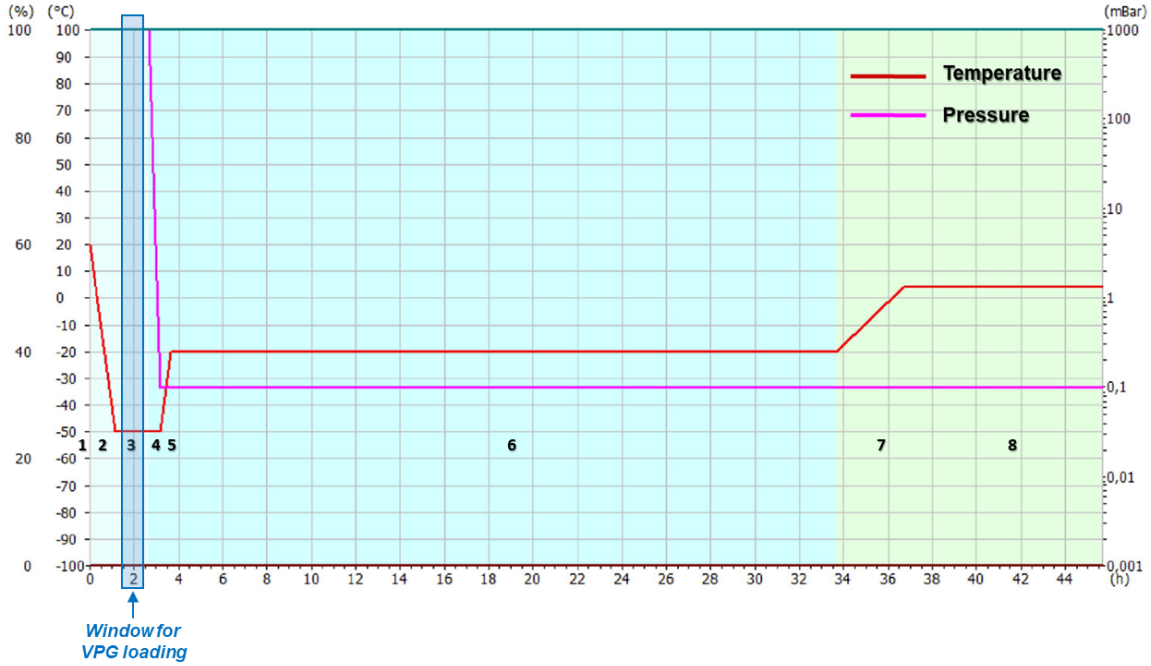

Figure 2-5 Overview of the program of VPG Freeze-drying

First, chamber temperature was equilibrated to 20 °C, and empty vials were placed as placeholders for the immediately frozen VPG formulations surrounded by vials filled with 3 mL of buffer. For immediate freezing, the VPGs were immersed in liquid nitrogen for 1 min. After cooling to - 50 °C using a ramp of 1 °C/min, placeholders were exchanged with the immediately frozen VPGs. The chamber pressure was reduced to 0.100 mbar, and shelf temperature increased to - 20 °C for the primary drying. After 30 hours primary drying in - 20 °C, the temperature was increased to 4 °C for 8 hours secondary drying. Last, the vials were purged with Nitrogen to 600 mBar before closing the rubber stopper completely.

2.2.9.2 Droplet VPG freeze drying

The VPGs were filled into syringes (10 mL (12mL) NORM-JECT®), and then fixed on a syringe pump (KDS-220-CE, KD Scientific Inc, USA). Different lipid concentration VPGs were dropped into the glass vials immersed in liquid nitrogen drop by drop (flow rate: 1mL/min) [13]. Then the freeze drying protocol (see Figure 2-5) was applied.

2.2.9.3 The reconstitution of freeze-dried VPGs

Highly purified water was added for reconstitution, in an amount (weight) corresponding to the water lost during the freeze-drying. Then the glass vials were left standing for 10 min stabilization, and after that, the samples were mixed by a stirrer for 45 min (from 100 rpm to 300 rpm, the rotation rate was rising up step by step slowly), to obtain the reconstituted VPGs (Lyo-rec-VPG).

2.2.9.4 Moisture analysis by Karl Fischer titration

Karl Fischer titration was used to determine residual water contents after freeze-drying. Between 10 and 30 mg of each formulation was used and the samples were prepared in a glove box filled with pressurized air with a relative humidity of less than 10%. The samples were then placed in an oven with 100°C to enable the fast extraction of water. The headspace moisture is transported into a coulometric Karl Fischer titrator (Aqua 40.00; Elektrochemie Halle, Halle, Germany). Results are calculated as relative water content (w/w).

2.2.9.5 Peroxidation analysis of phospholipids

Lipid peroxidation is the degradation of lipids that occurs as a result of oxidative damage and is a useful marker for oxidative stress. Polyunsaturated lipids are susceptible to oxidative stress, typically by reactive oxygen species, resulting in a well-defined chain reaction with the production of end products such as malondialdehyde (MDA) [14]. The peroxidation of the phospholipids was quantified by using a Lipid Peroxidation (MDA) Assay Kit (Cat No. MAK085, Sigma-Aldrich Co. LLC). During the kit measurement, the lipid peroxidation is determined by the reaction of MDA with thiobarbituric acid to form a fluorescing (λ_{ex} = 532 nm / λ_{em} = 553 nm) product, proportional to the MDA present [14].

2.2.9.6 Frozen VPG storage method.

The VPG or mAb loaded VPG were prepared, and filled into the glass vails (Injection Bottles 10R, with 20 mm neck, 45.0*24.0/1.00mm, MGlas AG), the glass vails with samples were put into the sample boxes and placed in 4°C before freezing. And then, the sample boxes were placed into either -20°C or -80°C Freezer separately. After certain time points, the samples will be taken out for measurements.

2.2.10 Antibody labelling and analysis

The antibody was dialyzed into 20mM PBS to remove the primary amines in the buffer, that are not compatible with the NHS-labeling reaction, because they will react with the NHS-ester moiety (*N*-Hydroxysuccinimide (NHS)-ester). The fluorescein for conjugation was NHS-Fluorescein (5/6-carboxyfluorescein succinimidyl ester) (Prod # 46409, Thermo Scientific™) [15].

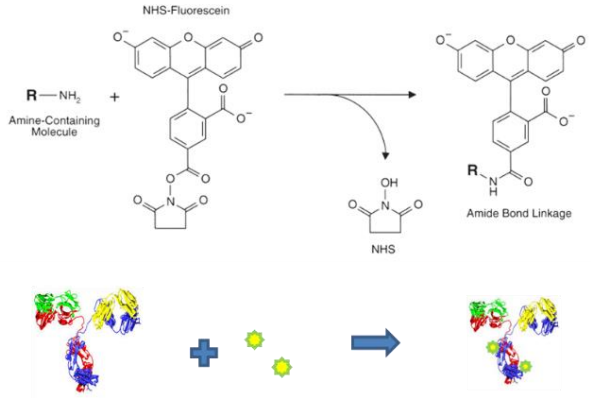

Figure 2-6. The scheme of the antibody labeling with the NHS-Fluorescein [15].

After the buffer exchanging, 500 mg of antibody (in 20 mM PBS) were labeled with NHS-Fluorescein (dissolved in DMSO), by mixing and incubating for 1 hour at room temperature. After that, the solution was dialyzed to remove the free fluorescein. Then, after 3 days of dialysis, the mAb-NHS was obtained. The absorbance of the sample was measured by the Spectrophotometer (280nm and 493nm). The protein concentration was calculated by the following formula. ($\varepsilon_{protein}$ = protein molar extinction coefficient (e.g., the molar extinction coefficient of IgG is ~210,000 M^{-1} cm^{-1}), and $A_{max} = A_{493}$, CF = Correction factor= A_{280}/A_{max}=0.3).

$$\text{Protein concentration (M)} = \frac{A_{280} - (A_{max} \times CF)}{\varepsilon_{protein}} \times \text{dilution factor}$$

The final degree of labeling was calculated by the following formula (ε_{fluor} = 70,000 (NHS-Fluorescein molar extinction coefficient))

$$\text{Moles fluor per mole protein} = \frac{A_{max} \text{ of the labeled protein}}{\varepsilon_{fluor} \times \text{protein concentration (M)}} \times \text{dilution factor}$$

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Chapter 3 Extraction and analysis of a monoclonal antibody in high lipid concentration gel formulations

3.1 Introduction

Due to the amphiphilic nature of the phospholipids, vesicular phospholipid gels (VPGs) show good performance both in the hydrophobic and hydrophilic molecular drug encapsulation. The semi-solid dispersions are mainly composed of phospholipids, the lipid layers can encapsulate the hydrophobic molecules, and the internal “polyhedral” structures can shield all ingredients from the aqueous phase during gel preparation. These high lipid containing depot formulations were more and more used as protein drug sustained release systems because protein stability was maintained in these formulations in previous work. The protein loaded VPG formulations have a high potential for cancer therapies or the treatment of chronic diseases in the future. However, feasible protocols for protein characterization are not available yet [1,2].

In previous research, different methods have been developed for drug loaded VPGs, and most were organic solvent extraction[3-5] for quantitative analysis, especially for small molecules. Different from these small molecules, protein drugs are sensitive to surrounding chemical, physical, and biological factors. In biomolecules analysis, the bio-activity and bio-affinity analysis is a very important part during the formulation efficacy and stability study, and the organic solvents are not compatible with those assays [6].

Here, after comparison of different methods, a novel method for the extraction and analysis of a monoclonal antibody (mAb) from high lipid concentration gel formulations has been developed, and the structural stability and bio-affinity of the protein was tested. The lipid removal agent (LRA) is a material that can remove the lipids from samples by physical adsorption, which was used for bio-products purification in biotechnological applications, and also was used for the lipid removal from the plasma samples for protein analysis in pharmaceutical research[7].

3.2 Antibody loaded VPG formulations preparation and extraction

3.2.1 Antibody loaded VPG formulation preparation

Different formulations of various mAb and lipid ratios were prepared to test this method, which included three different lipid content VPGs (30%, 40%, 50% lipid concentration) with different antibody (LMU-1) concentration (0%, 1%, 5%, 10% protein) (Table 3-1). All samples were prepared by Dual Asymmetric Centrifugation, (DAC SpeedMixer^{TM}) with 3500 rpm centrifugation speed and 45 min at 2~8°C.

	0% mAb in VPG (w/w)	1% mAb in VPG (w/w)	5% mAb in VPG (w/w)	10% mAb in VPG (w/w)
30% lipid content VPG (w/w)	**30%VPG**	**1%mAb@30%VPG**	**5%mAb@30%VPG**	**10%mAb@30%VPG**
40% lipid content VPG (w/w)	**40%VPG**	**1%mAb@40%VPG**	**5%mAb@40%VPG**	**10%mAb@40%VPG**
50% lipid content VPG (w/w)	**50%VPG**	**1%mAb@50%VPG**	**5%mAb@50%VPG**	**10%mAb@50%VPG**

Table 3-1. The formulations in the antibody loaded VPG extraction study

3.2.2 The antibody loaded VPG extraction method

In the pre-tests, the LRA could not separate the lipid and protein completely. Because of that, 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) was added to the extraction method as surfactant for protein and lipid separation. Based on the standard protocol and previous research, different amount of CHAPS and LRA ratios were tested to purify the mAb (Table 3-2).

Because one purpose of this research is the development of an improved method for the extraction of the antibody from formulations with a super high lipid concentration, the final concentration of the active protein and the lipid are the core parameters. As a high concentration of active protein and low concentration of lipid is desired, the step of adding the detergent can be seen as a key point. Compared with protocol and other research works, different amount of CHAPS and LRA groups were designed for purer mAb. This is the reason why 50 µL and 100 µL CHAPS solution was tested for different samples and 40 mg or 60 mg LRA powder were added during the extraction (Table 3-2).

	50 µL CHAPS (4%w/v)	100 µL CHAPS (4%w/v)
40 mg LRA	**50CHAPS-40LRA**	**100CHAPS-40LRA**
60 mg LRA	**50CHAPS-60LRA**	**100CHAPS-60LRA**

Table 3-2. The surfactant and lipid remove agent test groups

After experiment groups were set, the extraction experiments were started (Figure 3-1). 20 mg of the antibody loaded VPG (mAb@VPG) (Table 3-1) was placed into a 2 mL micro-centrifugation tube, then mixed with 930 µL 20mM PBS buffer using the Capmix™ for 2 second and afterwards either 50 µL or 100 µL CHAPS water solution (4% w/v) and either 40 mg or 60 mg LRA powder were added. The respective extraction samples are defined as 50CHAPS-40LRA, 50CHAPS-60LRA, 100CHAPS-40LRA and 100CHAPS-60LRA (Table 3-2).

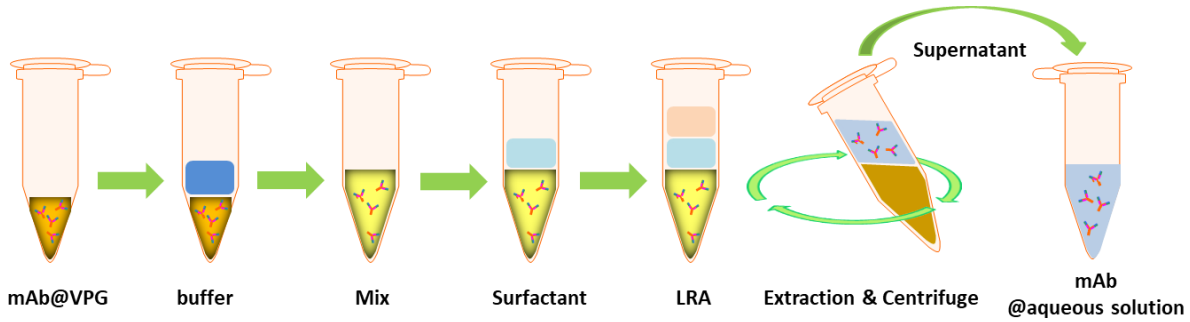

Figure 3-1. Scheme of operation steps for mAb extraction from VPG.

CapMix™ was used to mix each of the samples three times for 1 sec. at a time. A 1 mg/mL mAb solution was used as positive control, the negative control consisted of 20 mM PBS. All samples were incubated for one hour at 37 °C and a shaking rate of 90 rpm. Afterwards all tubes were centrifuged at 4 °C, 10000 g, for 10 min. After centrifugation, the supernatant (about 600 µL) was removed by pipetting and the extracted mAb solutions were stored in 1.5 mL tubes for the following measurements.

3.3 Results and discussion

3.3.1 The lipid and protein concentration after extraction

Phospholipids are the main component of the mAb@VPG formulation, which should be removed after extraction for easier analysis of the protein. The protein (mAb) is the active drug and should be sufficiently concentrated after extraction for further analysis.

After extraction with different extraction methods, the lipid and protein residual concentrations in the extracted samples were measured separately. The lipid concentration was measured using the phospholipid test assay kit (LabAssay^{TM}), and the protein residual concentration was measured using the Nanodrop^{TM}. The results are given in Figure 3-2.

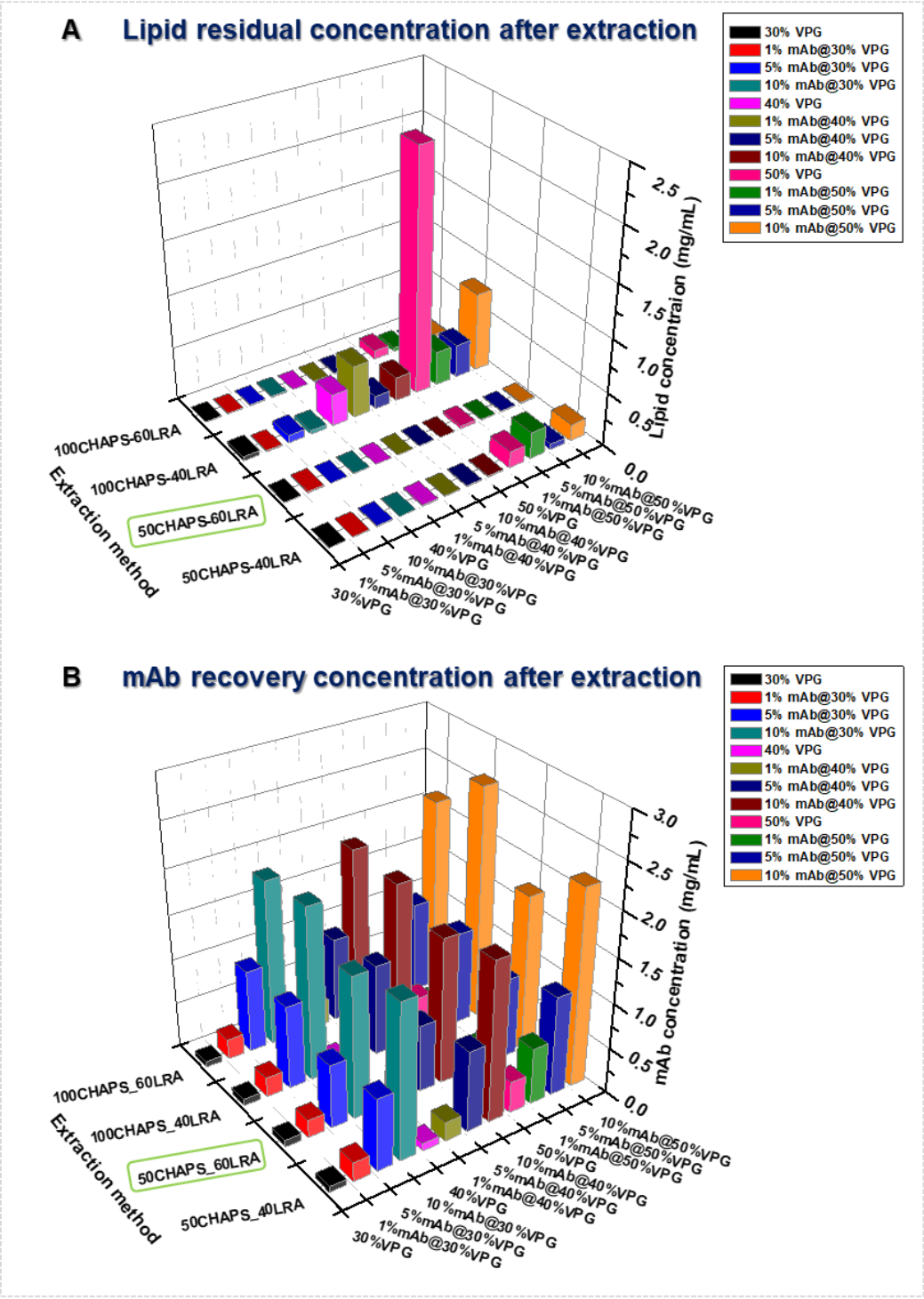

Figure 3-2. The Concentrations of mAb and Lipid after Extraction. (A) Lipid residual concentration after extraction. (B) mAb recovery concentration after extraction.

As seen in Figure 3-2 (A), the 50CHPAPS-60LRA combination had the lowest lipid concentration, whereas the lipid concentrations of samples prepared by other methods are high. Thus, the 50CHPAPS-60LRA (green markers) combination results in the best purification. The highest lipid residual concentration in all groups with this treatment was the 50%VPG groups, which less than 0.270%, about 0.027mg/mL, and the average residual rate of all groups was about 0.098%.

At the same time, as seen in figure3-1 (B), the concentration of mAb was sufficiently high, for analysis. However, the LRA adsorbed both lipids and protein molecules. Although the adsorption capability of LRA for lipids was much stronger, there was still some protein adsorbed by LRA. The adsorption did not necessarily have a negative impact to VPGs with higher protein content but for formulations with low protein concentrations, the protein yield for analysis was too low. Because the lipid should be removed as good as possible, during the 50CHPAPS-60LRA combination, the lowest mAb (protein) recovery rate was 70.0% (5%mAb@30%VPG), and the average recovery rate of all groups was about 87.3%, which was enough for analysis for further analysis. Hence, the 50CHPAPS-60LRA combination was selected for the following mAb@VPG extraction operation and protein stability testing.

There still some protein loss in the extraction operations, and the possible factor was the LRA also has slight adsorption to protein molecules. Some other adsorption powder or detergents could also be test in the future for better purification result with higher protein recovery.

3.3.2 The protein structural stability test

3.3.2.1 The protein structural stability analysis by SDS-PAGE

Because the tertiary and quaternary structure of the protein, as well as the bio-activity, are very sensitive to physical and chemical factors, the protein was analysed by SDS-PAGE for structural analysis (Figure 3-3). All electrophoresis bands of the samples matched to the bands of the reference, the stock antibody solution. The SDS-PAGE did not indicate any aggregation that may have occurred during extraction.

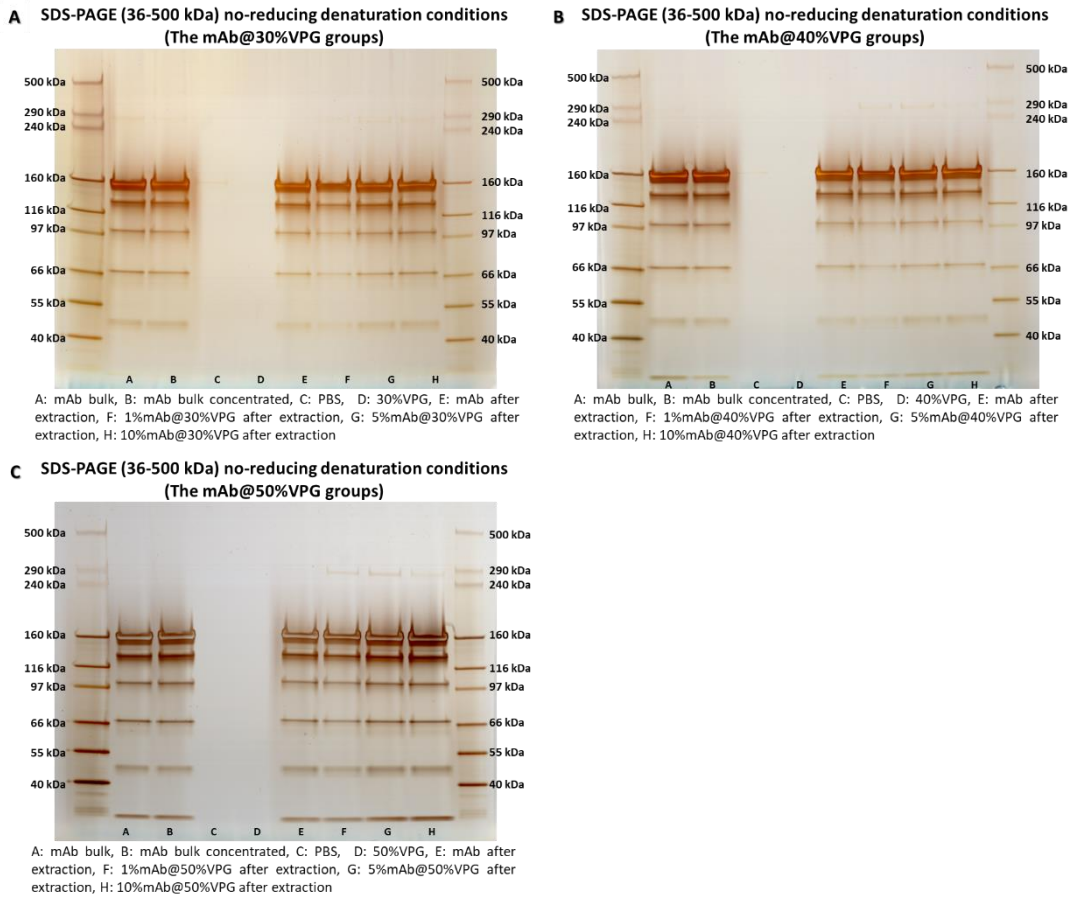

Figure 3-3. SDS-PAGE for mAb extraction from (A) 1%, 5%, 10% mAb@30%VPG; (B) 1%, 5%, 10% mAb@40%VPG; (C) 1%, 5%, 10% mAb@50%VPG.

3.3.2.2 Chromatographic analysis of protein stability by Size Exclusion High Performance Liquid Chromatography (SE-HPLC)

The protein monomer content was determined by SE-HPLC. For quantitative analysis, a dilution series of the antibody stock solution was prepared as a standard curve. The protein content in the extracted samples was then determined by comparison to the standard curve. To prevent block of the column by insoluble particles, the sample solutions were tested after centrifugation (10000g 10min). The detailed methods are described in Chapter 2. The chromatograms of SE-HPLC was placed in Figure 3-4, and the main peaks of protein were clear (yellow arrow).

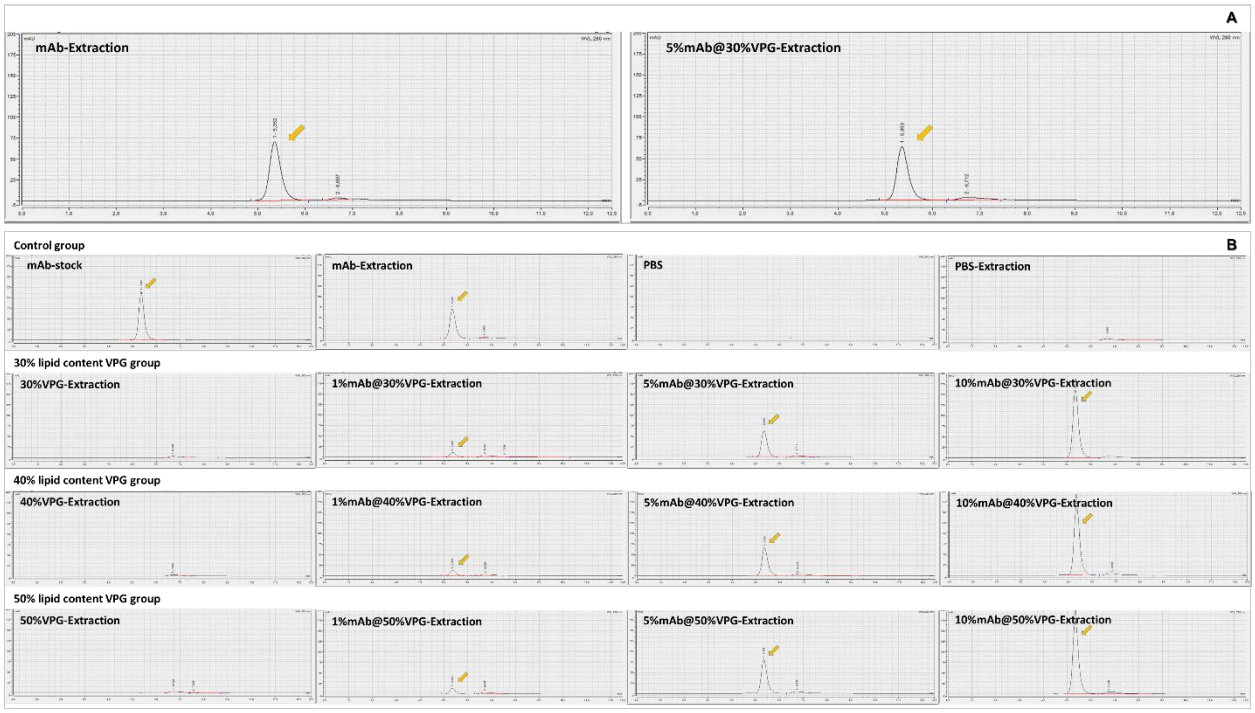

Figure 3-4. Chromatograms of SE-HPLC for mAb extracted from mAb@VPG. A, the chromatograms for mAb after extraction, and 5%mAb@30%VPG after extraction. B, the chromatograms for all measurement groups.

The protein monomer recovery concentrations (based on the main peak) are shown in Table 3-3 and Figure 3-5 A. The determined concentration was compared to the theoretical concentration of mAb within the VPG, according to the protein concentration used for the preparation, the recovery rate of monomer protein was got (Figure 3-5 B).

SE-HPLC analysis of mAb after extraction			
Sample	**Ret.Time (min)**	**mAb con. (mg/mL)**	**mAb Recovery rate (%)**
PBS	[5.23 - 5.50]	0.000	---
PBS_Extr	[5.23 - 5.50]	0.000	---
1mg/mL_mAb	[5.23 - 5.50]	0.969	96.9
1mg/mL_mAb_Extr	[5.23 - 5.50]	0.643	64.3
30%VPG-Extraction	[5.23 - 5.50]	0.000	---
1%mAb@30%VPG-Extr	[5.23 - 5.50]	0.110	54.9
5%mAb@30%VPG-Extr	[5.23 - 5.50]	0.670	67.0
10%mAb@30%VPG-Extr	[5.23 - 5.50]	1.474	73.7
40%VPG-Extraction	[5.23 - 5.50]	0.000	---
1%mAb@40%VPG-Extr	[5.23 - 5.50]	0.145	72.3
5%mAb@40%VPG-Extr	[5.23 - 5.50]	0.993	99.3
10%mAb@40%VPG-Extr	[5.23 - 5.50]	1.570	78.5
50%VPG-Extraction	[5.23 - 5.50]	0.000	---
1%mAb@50%VPG-Extr	[5.23 - 5.50]	0.155	77.7
5%mAb@50%VPG-Extr	[5.23 - 5.50]	0.765	76.5
10%mAb@50%VPG-Extr	[5.23 - 5.50]	1.571	78.5

Table 3-3. mAb extracted from mAb@VPG by SE-HPLC

In the SE-HPLC. the recovery rate of stock mAb after extraction was 64.3%, and mAb loaded VPG were between 54.9% to 99.3%, and the average value was 75.4%.

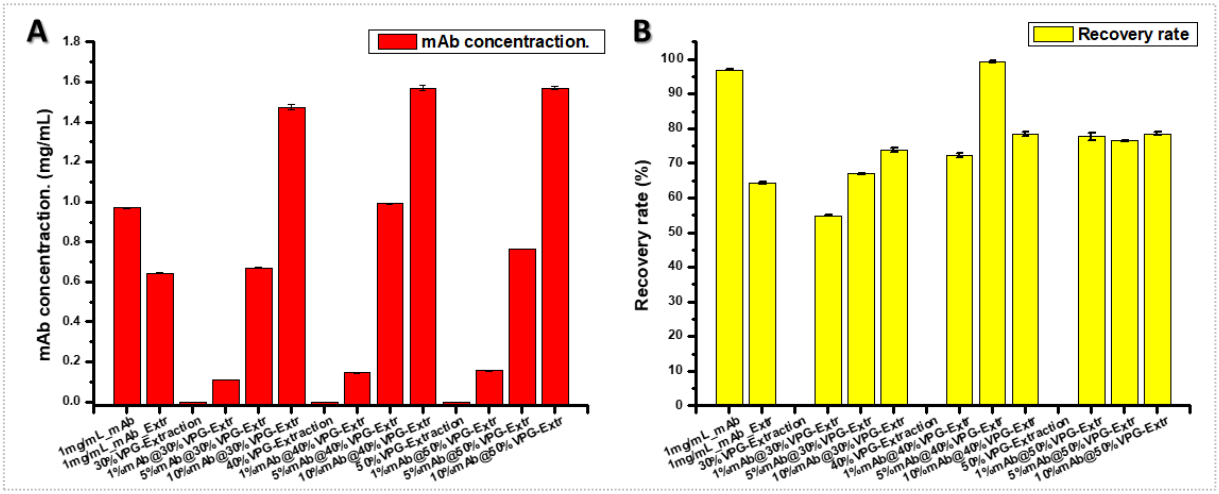

Figure 3-5. mAb extraction from mAb@VPG by SE-HPLC, (A) Absolute concentration of monomer antibody after extraction, (B) Recovery rate of monomer antibody compared to the theoretical concentration after extraction.

The SE-HPLC measurement show that only small amounts of, fragments and aggregates are formed, (most samples less than 1 %). It seems that the extraction method does not disturb the protein structure. However, it may also be possible that fragments and aggregates are removed during the extraction by the LRA.

From the quantitative analysis of SE-HPLC, the protein remains monomeric after extraction and that means the extraction methods are gentle for the antibody. The concentration of monomer antibody after extraction treatment was high enough for the further analysis.

3.3.3 The protein chemical stability test by IEX-HPLC

The detection of charge variants of the mAb after the extraction is an indicator for chemical instability, and therefore IEX-HPLC is a feasible stability indicating method to prove that the extraction gentle for the mAb. The chromatograms of mAb loaded VPG was placed in Figure 3-6, and the main peaks were stable in all groups (green arrow). The results were calculated and displayed by comparison to the mAb stock samples (Figure 3-7).

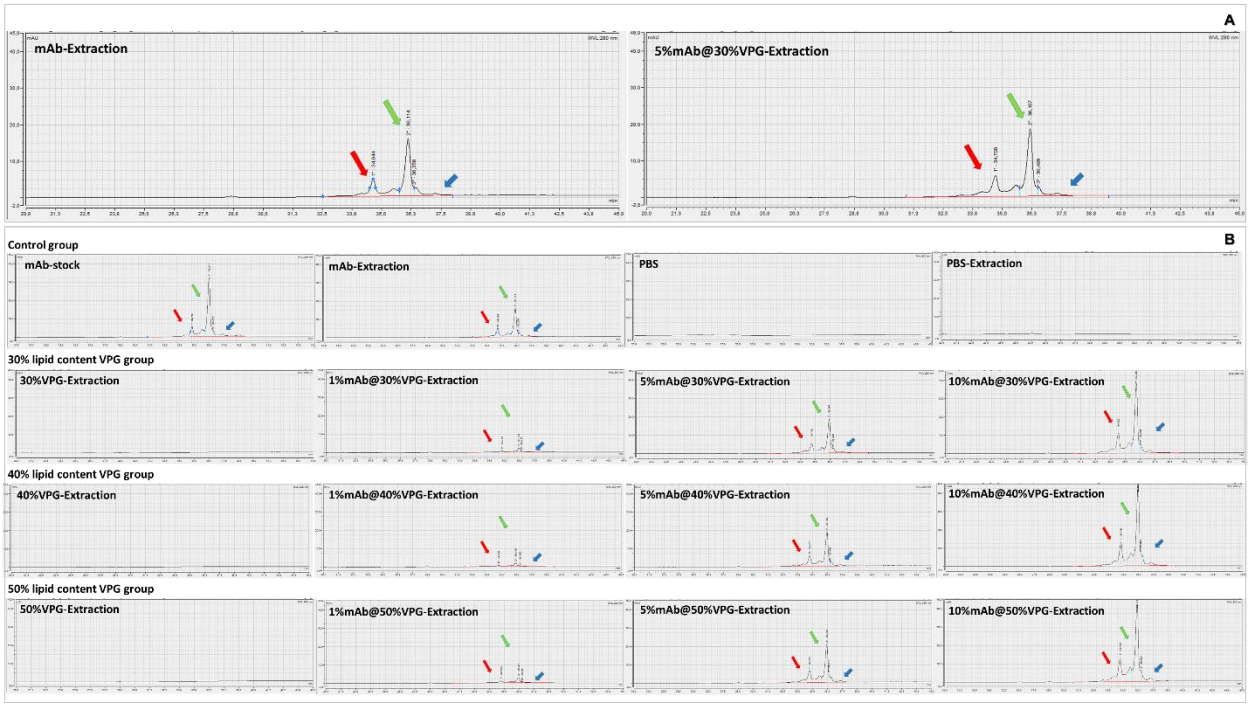

Figure 3-6. Chromatograms of IEX-HPLC for mAb extracted from mAb@VPG. Red arrow: Acidic species; Green arrow: Main Peak; Blue arrow: Basic species. A, the chromatograms for mAb after extraction, and 5%mAb@30%VPG after extraction. B, the chromatograms for all measurement groups.

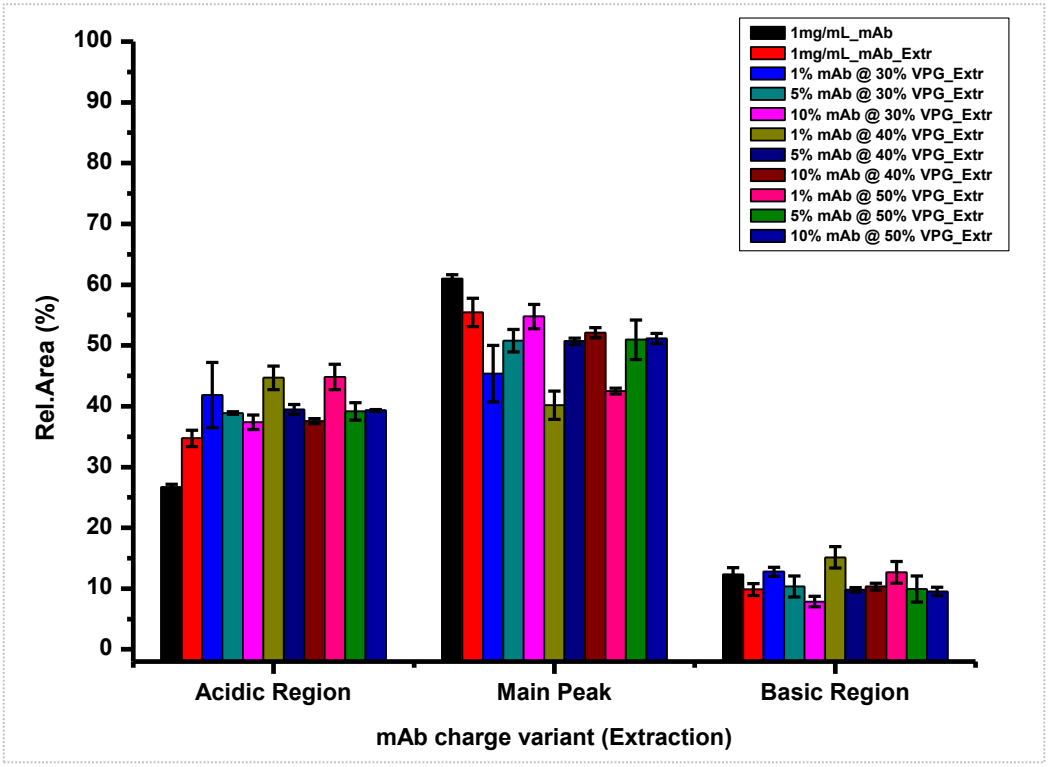

Figure 3-7. mAb extraction from mAb@VPG by IEX-HPLC.

The chemical stability after extraction was generally good, and especially in the high lipid content formulation. The acidic region has slightly increased in the low protein concentration (1%mAb@VPG) groups.

3.3.4 The protein conformation stability test by Near UV Circular Dichroism (CD)

Circular Dichroism is a very powerful method to investigate the tertiary structure of a protein. Because there was still small amount lipid in the extracted solution, FTIR could not resolve the mAb structure, but the near-UV CD spectroscopy (250-320 nm) worked well for the extracted mAb@VPG samples[8]. The high protein content VPG formulations (5% and 10% mAb@VPG) showed the typical peaks of the native mAb which includes a positive peak around 295 nm and several negative peaks between 280 and 250 nm (Figure 3-8, A B)[9]. However, the CD-measurements did not work well for the low protein content formulations (1%mAb@VPG), which because the signal form protein was low (Figure 3-8, C).

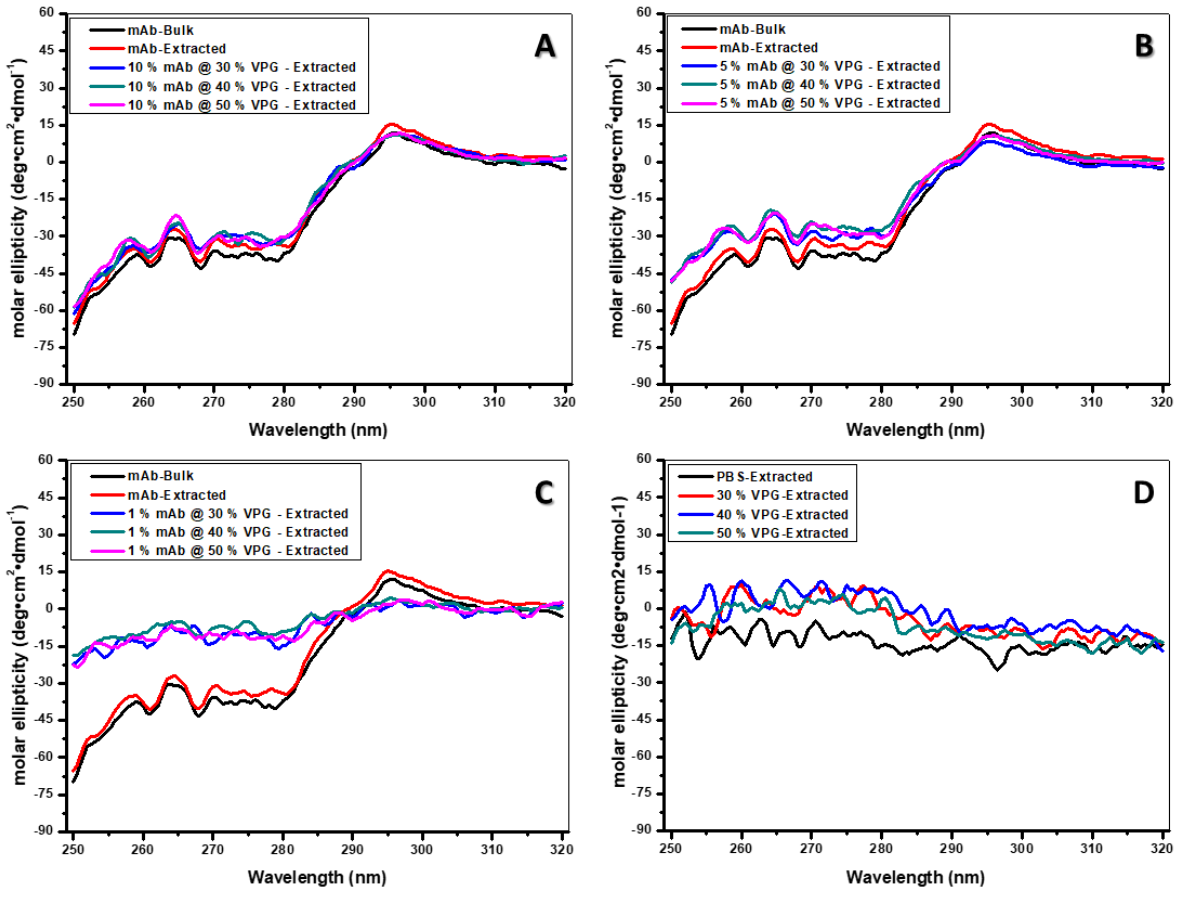

Figure 3-8. Circular Dichroism of mAb extracted from mAb@VPG, (A) 10% mAb@VPG group; (B) 5% mAb@VPG group; (C) 1% mAb@VPG group; (D) negative control groups.

Based on the results of CD spectroscopy, the proteins conformational stability after extraction could be shown, which means the antibody structure was still native after extraction.

3.3.5 The bio-affinity stability analysis of mAb by Bio-layer interferometry

The ability of bio-layer interferometry (BLI) to detect structurally altered and/or aggregated species of pharmaceutically relevant proteins has been demonstrated earlier [10,11], and the protein concentration could also be quantitatively analysed [11,12]. In the investigation of the extraction of mAb@VPG, the BLI was used for the Fc and Fab region affinity analysis. The Protein A Biosensors (Fc, 18-5010) was used for Fc region, and the Anti-Human Fab-CH1 2nd Generation (Fab2G, 18-5125) Biosensors was used for Fab region. Both Fc and Fab2G were tested for the samples to prove the bio-affinity stability.

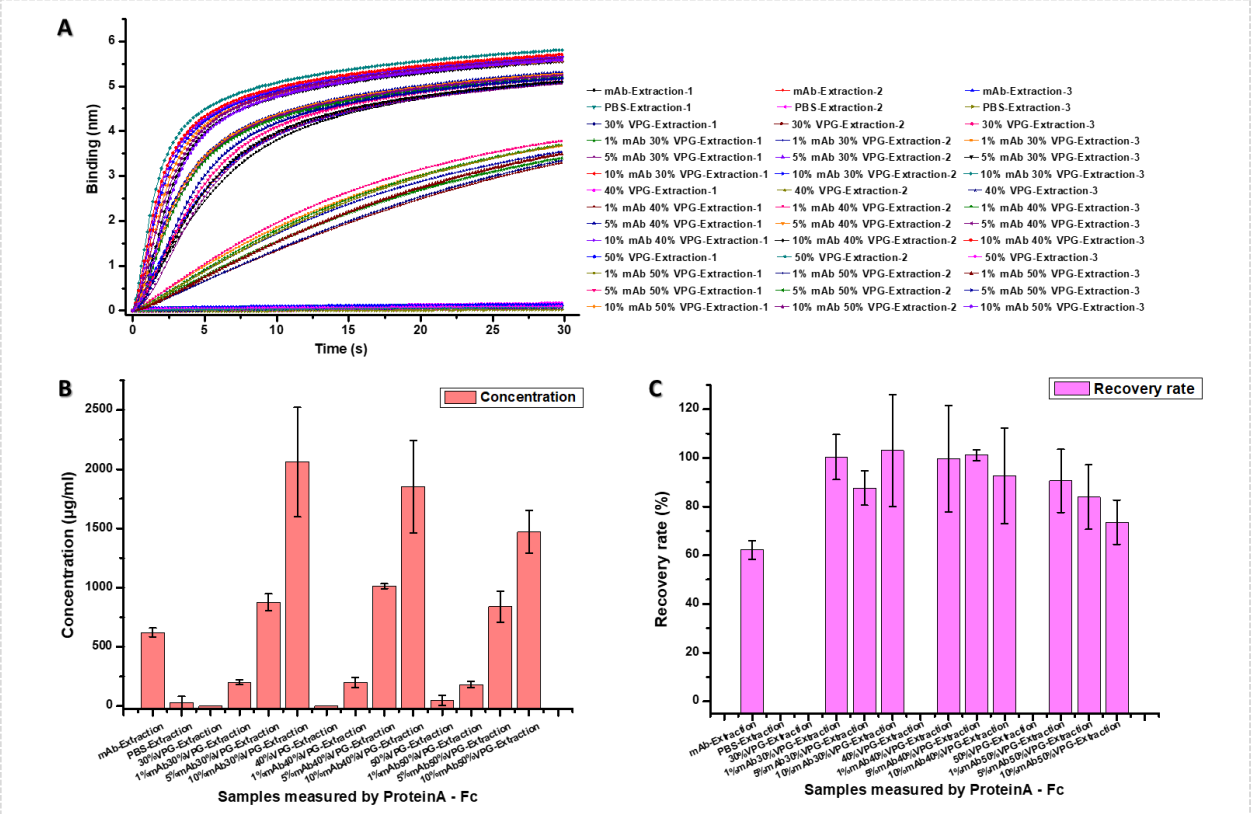

Figure 3-9. The protein (mAb) Fc region affinity measurement after extraction from VPG; (A) Fc region binding curves of mAb after extraction from VPG formulation; (B) The Fc region active protein concentration of mAb after extraction from mAb@VPG; (C) The Fc region active protein recovery rate of mAb after extraction from mAb@VPG.

The association curves of Fc were shown in Figure 3-9 A. By comparison to standard curves, the concentrations of Fc were calculated (Figure 3-9, B), and the recovery rate of mAb regions were received by calculation using the theoretical concentration, the recovery rate of stock mAb after extraction was 62.16%, and mAb loaded VPG were between 73.56% to 101.15% (Figure 3-9, C).

The association curves of Fab is shown in Figure 3-10 A. By comparison to standard curves, the concentrations of Fab was calculated (Figure 3-10, B), and the recovery rate

of mAb regions were received by calculation using the theoretical concentration (Figure 3-10, C). the recovery rate of stock mAb after extraction was 74.0%, and mAb loaded VPG were between 77.8% to 105.0%.

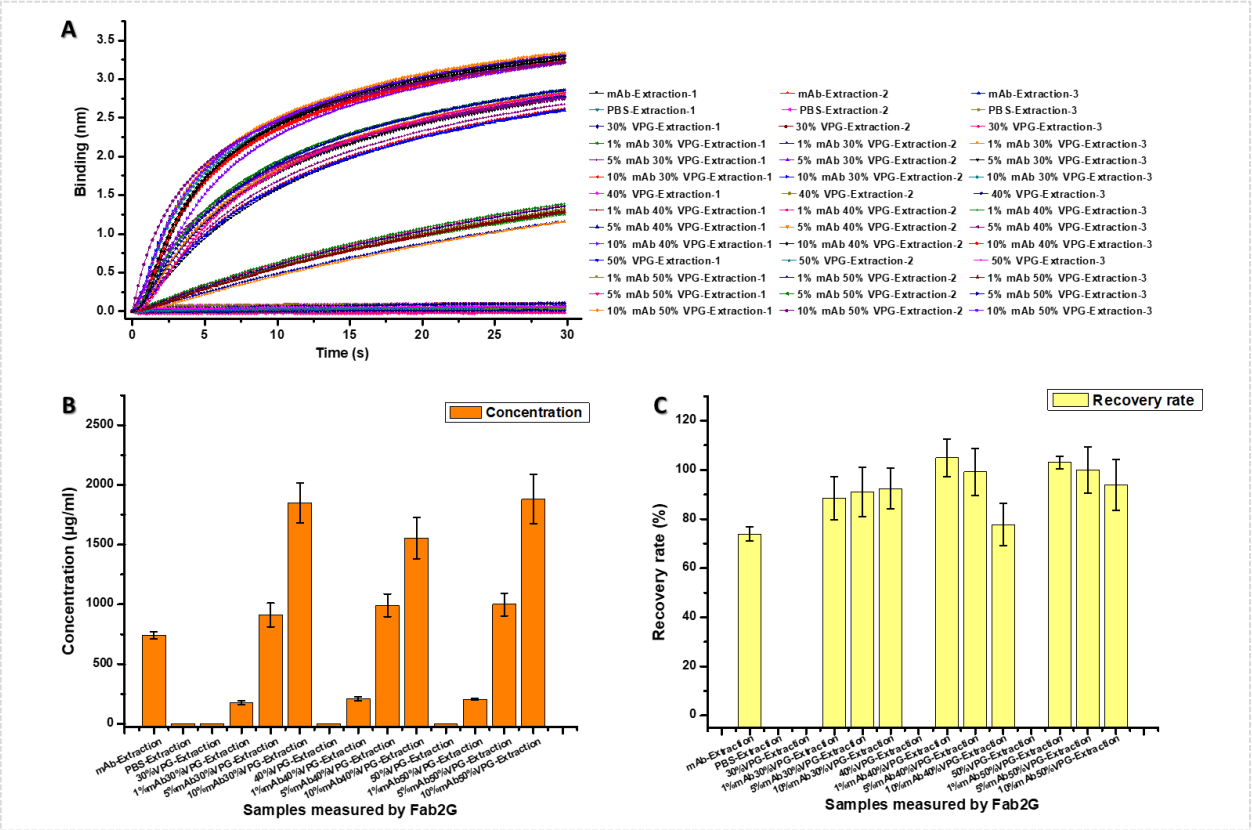

Figure 3-10. The protein (mAb) Fab region affinity measurement after extraction from VPG; (A) Fab region binding curves of mAb after extraction from VPG formulation; (B) The Fab region active protein concentration of mAb after extraction from mAb@VPG; (C) The Fab region active protein recovery rate of mAb after extraction from mAb@VPG.

From the affinity analysis by BLI, both Fc and Fab region still have sufficient affinity, so those regions were stable enough during the extraction operation, and the main biological function of mAbs was preserved. Compared with quantitative analysis by SE-HPLC, the measurement value from BLI of mAb loaded VPG groups' value were slightly higher, and the possible reason was the centrifugaion before the sample injection in SE-HPLC, which removed some large particles, and also could have removed small amounts of protein. At the same time, there was slightly nonspecific binding happening during BLI measurement for mAb loaded VPG after extraction. Hence, the extraction method using CHAPS and LRA had no negative impact on the protein bio-affinity performance.

3.4 Conclusion

In the optimization experiments for the development of a new extraction method, different CHAPS and LRA contents were tested, and the residual concentrations of lipid and protein were measured. For the series of mAb loaded VPG samples, the 50CHPAPS-60 LRA combination was selected because of the best ratio of protein (recovery rate: 87.3%) to lipid (residual rate: 0.098%). The protein residual concentration in the high lipid VPG formulation was higher than in the low lipid formulations. The LRA powder adsorbed both lipid and protein, but the adsorption to lipid was stronger than to the protein, which means higher concentrations of lipid prevent unspecific adsorption of protein. The protein structural stability was measured by SDS-PAGE and SE-HPLC. Both methods showed feasible stability of the protein after extraction. Also the chemical stability by IEX-HPLC indicate a preserved stability after extraction. By CD analysis, the tertiary structure of the mAb in different protein loaded VPG groups was shown,

The biological affinity tests for Fab and Fc of the used mAb by BLI was a very important part to investigate whether the extraction operation was gentle enough for the antibody. Compared to the mAb stock solution, both Fc (recovery rate: 91.4%) and Fab (recovery rate: 94.6%) regions kept good affinity capability after extraction.

A novel, easy and optimized mAb@VPG extraction and analysis method was developed in this work. The protein structural stability and biological activity could be effectively measured and this method is gentle enough for the mAb drug in high concentration lipid formulations. This extraction method can be used for further investigations of long term stability of mAbs in the VPG.

3.5 Reference

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Chapter 4 *In vitro* VPG erosion and release studies

4.1 Introduction

4.1.1 Development of a new release test model for VPGs

The Flow-through cell model was developed in 1998 by C. Tardi et al., and the model was designed with one donor compartment inside the release cell. The donor compartment is a long channel, with a semicircular cross-section that facilitates the laminar flow of the acceptor medium. Several other models have been used for VPG investigation. However, so far release models for VPGs are not good enough. First, a small volume and tissue mimicking properties are desirable, and not well provided by the existing models. Second, the erosion and diffusion release mechanisms are unclear. These models were tailored to deliver “any” release type, and it is still unclear whether free or liposome bound drug is released. Third, the setup is very complex, which includes syringe pumps, water bath, and refilling of the syringe every few days, which is not suitable for long-term release experiments. Finally, the old test systems lack correlation with *in vivo* PK studies.

On the other hand, some researchers used a dialysis bag in the *in vitro* release experiment, such as Zhang et al[1]. This method is faster and easier to setup than the flow through release cell model, and lipid particles / liposomes not were not released from the VPG samples.

4.1.2 Membrane selection for the new release test model

In the traditional “membrane free” release models for VPGs, particles up to or above the micron size can release from the VPGs, which are eroded from the VPG surface during the passage of the release buffer. This phenomenon was avoided by using the dialysis bag release model, where particles could not be released from the bag. Hence, some membrane could be set into the release cell model to control the particle release from the VPG bulk. Using this approach, more accurate release results could be obtained, during the *in vitro* experiments. According to the research of Foster et al, Storm et al and Goins et al. [2-4], more than 63.3% of large liposomes (larger than 400 nm) will remain at the injection site after the subcutaneous (SC) injection over 24 hours, because for large size liposomes is difficult to penetrate tissue, and the lymphatic uptake

decreases with increasing liposome size [3,4]. Based on the results of Storm et al., the larger particles (larger than 40 nm) are not easily going to the blood circulation after subcutaneous administration. So materials and pore size of release membranes should could be a key parameters of a new model [3].

At the same time, the drug release matrix surrounding a VPG dose is an aqueous solution. VPGs are a gel formulation formed by phospholipids, and work as semisolid depot formulations for sustained drug release systems. According to these reasons, the material of the membrane should have enough water permeability to mimic tissue. Second, VPGs work as a depot drug delivery system, so the release time will be longer, and the filter membrane should have enough physical and chemical stability for several weeks. Third, the membrane pore size should be larger than 400 nm at least, and to allow free diffusion mode, the pore size of the release membrane should even be much larger to make sure the liposome can diffuse through the membrane like *in vivo,* and considering this, the pore size of the membrane during the following experiments should be larger than 1 µm. Considering the release model will be used for protein drug loaded VPGs, low protein binding is also important for the membrane selection.

4.2 Results and discussion of macromolecule loaded VPG erosion and release studies

4.2.1 The model drug for *in vitro* release studies

The model drug molecule should have enough stability for long term release experiments over several weeks, and the model substance should have the good chemical properties for quantitative analysis. FITC-BSA (fluorescein isothiocyanate labelled bovine serum albumin) is the first candidate for a model protein, because BSA alone cannot be analyzed with sufficient sensitivity. However, FITC-BSA is not stable enough for the long term release experiment[5]. For these reasons, the FITC-Dextran was selected as the model drug during the first phase of release model development. Dextran is carbohydrate with large molecular weight, and as early stage model substance during the protein formulation study. As the molecular weight of BSA is 66,463 Da, the FITC-Dextran (70 kDa) was selected for the first phase experiment. The Fluorescence signal of FITC is strong enough for quantitative analysis in the release experiments.

4.2.2 Development of an optimized flow through cell release test model

After the pretests with 1 μm pore size membrane, the release was very limited during the flow through cell (FTC) model studies, and then Cellulose Acetate membranes with a pore size of 5 μm were selected for the next release experiments. According the previous research, the FTC test model is a commonly used method for VPG formulations *in vitro* release investigation as SC administration mimicry [6-10]. The FTC was made of Teflon, with a donor chamber of 25mm in length and a rectangular cross-section of the donor chamber of 5×5 mm. There are two tubing adapters in two terminals of the donor chamber. The sample in the donor chamber releases the encapsulated drug directly into the above flowing buffer. Tubing adapters of the cells were connected with 60mL syringes with silicon tubing.

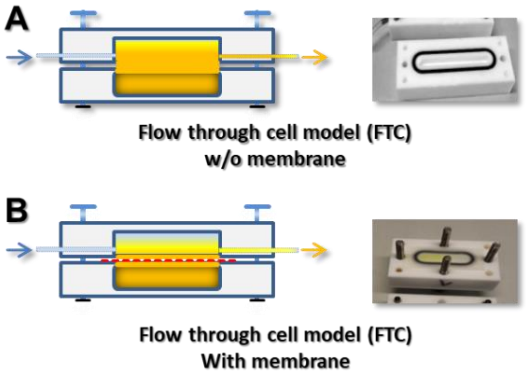


Figure 4-1 Schematic of Flow through cell model (FTC) w/o membrane (A), and Flow through cell model (FTC) with membrane (B).

A 10 mg/mL FITC-Dextran (20mM) PBS solution was used in the FTC with membrane release model (n=3), to act as a reference to estimate the diffusion hindrance caused by the membrane mimic tissue [11,12] (Figure 4-2), which drug concentration could arrive peak about in 1 day.

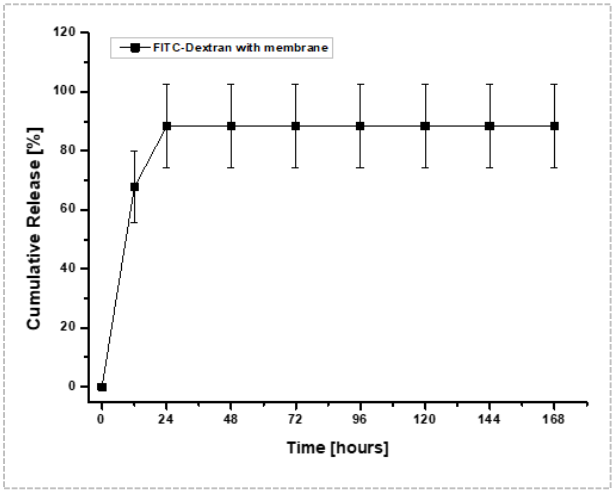


Figure 4-2 The release test for FITC-dextran in buffer solution release from FTC with membrane

The release behaviors of VPGs with 30%, 40% and 50% phospholipid content were measured (Figure 4-3) in FTCs with membrane and w/o membrane.

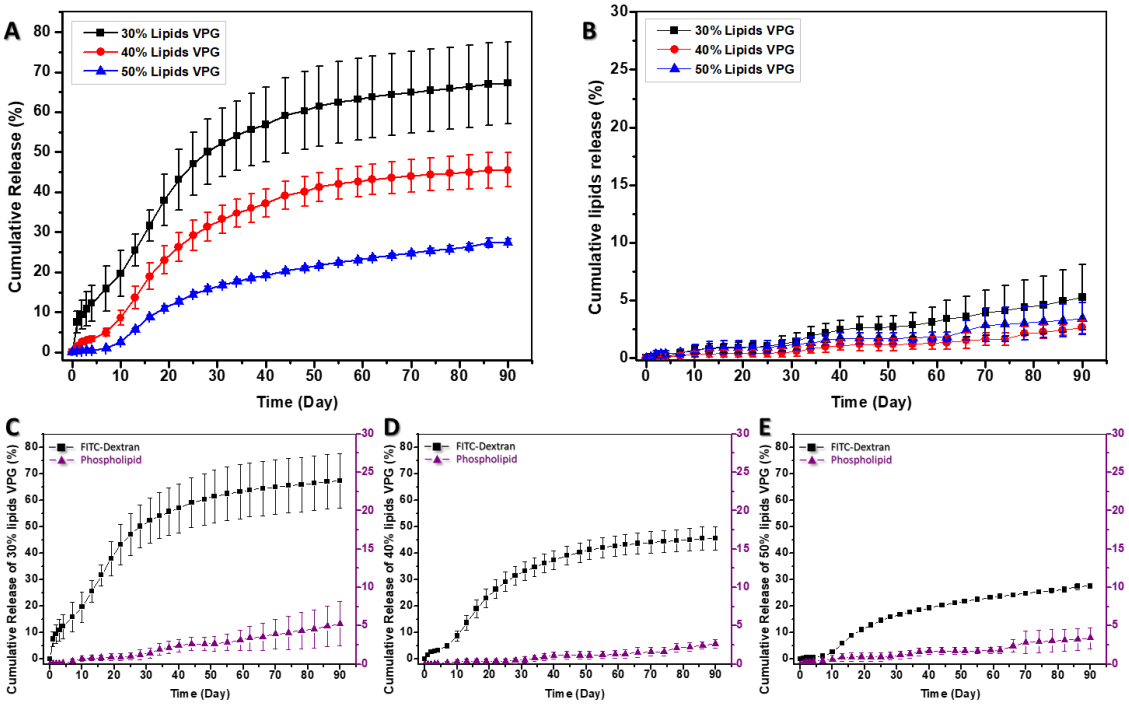


Figure 4-3 The FITC-Dextran and phospholipid release behavior in FTC release with membrane model. (A) Cumulative release of FITC-Dextran. (B) Cumulative release of Phospholipids. (C) The release of FITC-Dextran and lipid @ 30 % lipids VPG. (D) The release of FITC-Dextran and lipid @ 40 % lipids VPG. (E) The release of FITC-Dextran and lipid @ 50 % lipids VPG.

According to the results of the FTC with membrane (Figure 4-3), the FITC-Dextran could move through the membrane freely (Figure 4-2). At the same time, the lipid release was very slowly and most lipids were kept in the donor chamber by the membrane. The membrane could adjust the release status effectively, and mimics the release conditions in tissues more accurately. In the FTC w/o membrane (Figure 4-4), the FITC-Dextran

and lipid released together. And the release behavior of high lipid content groups were more close to the zero-order release as in the work of Brandl et al [6].

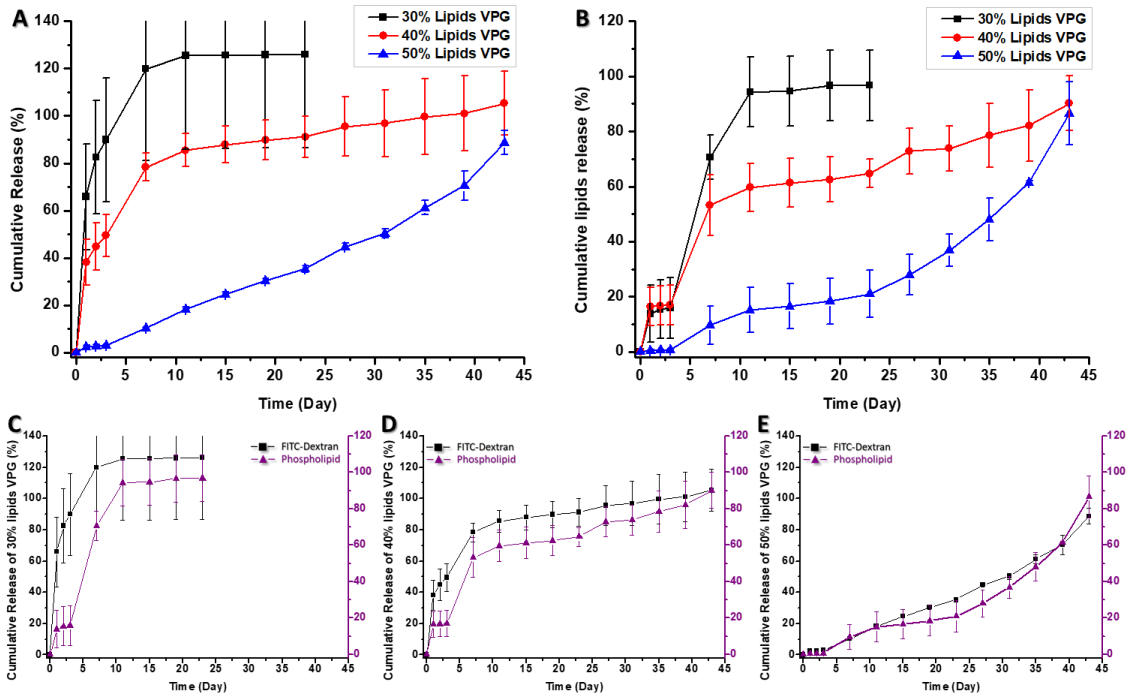


Figure 4-4 The FITC-Dextran and phospholipid release behavior in FTC release without membrane model. (A) Cumulative release of FITC-Dextran. (B) Cumulative release of Phospholipids. (C) The release of FITC-Dextran and lipid @ 30 % lipids VPG. (D) The release of FITC-Dextran and lipid @ 40 % lipids VPG. (E) The release of FITC-Dextran and lipid @ 50 % lipids VPG.

Because the release rates were very different in these two models, length of the release studies for FTC with membrane were set longer than for the FTC w/o membrane. The duration of the release experiment of FTC with membrane was set to 3 months, and for the FTC without membrane it was set to 1 ~ 1.5 months. At the final time point, most lipid was kept in the donor in FTC with membrane (Figure 4-5 A), while nearly everything had been released in the FTC w/o membrane, and the donor chambers of 30% lipid VPG groups were nearly empty (Figure 4-5 B). There was a short lag phase after the release beginning in lipid release measurement, and the possible reason was between the hydrophilic Dextran and hydrophobic phospholipid in the FITC-Dextran loaded VPG formulation. And the release behaviors of different VPGs in different release models were compared in Figure 4-24.

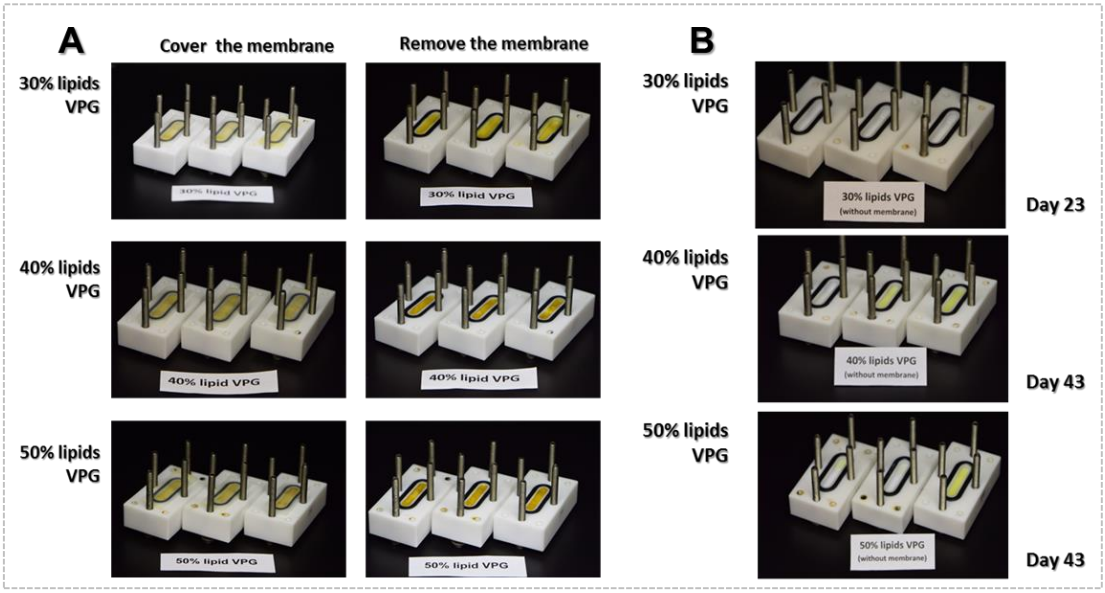


Figure 4-5. The final statue of VPG release experiment in FTC (A) with (5 μm pore size) membrane at Day 90, (B) without membrane in final time point.

Release buffer fractions from VPG release are shown in Figure 4-6, and the fraction from the FTC with membrane was more clear than the ones from the FTC w/o membrane, and the low lipid content group release visibly more FITC-Dextran then high lipid content groups.

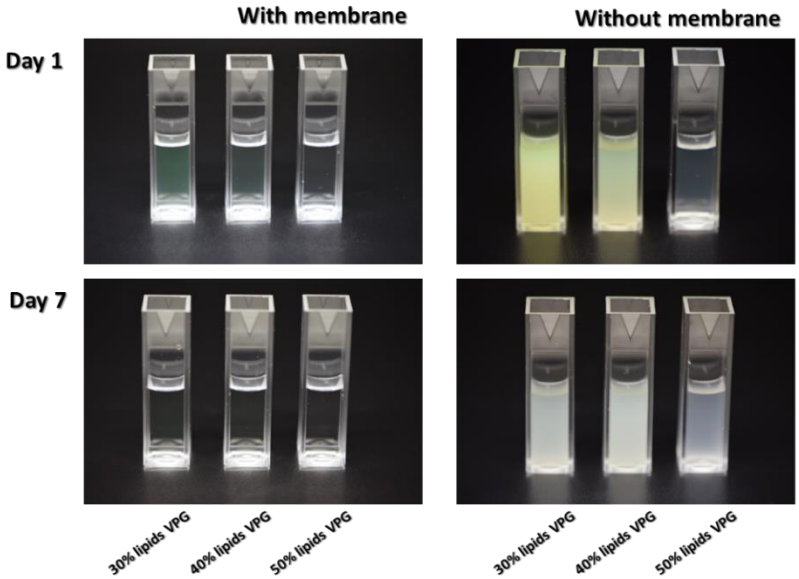


Figure 4-6 The VPG release fractions from different FTC release testing models at different time points.

The investigation of the status (free release or liposomal encapsulated) of the released drug is one purpose of the model study, and the particle size is a very important parameter to describe amount and nature of eventually release liposomes. Because the particle eroded from the VPG show an uneven size distribution, the z-average value is not suitable to analysis the VPG particles directly. The z-Average and Intensity Mean

value (Size) supplied basic information of mixed particles solution systems for the investigation of the VPG release behavior.

Higher derived count rates usually indicate higher concentration, larger particles, or higher concentration and larger particles. Therefore, the derived count rate (DCR) was used to estimate the total material mass released from the VPG. The VPG samples were diluted in 20mM PBS solution from 100 to 1000000 times (volume to weight ratio: 1980 µL PBS were used to dilute 20 mg VPG) and were analyzed by DLS measurement. According to the results (Figure 4-7), it can be shown that the DCR value decreases with increasing dilution.

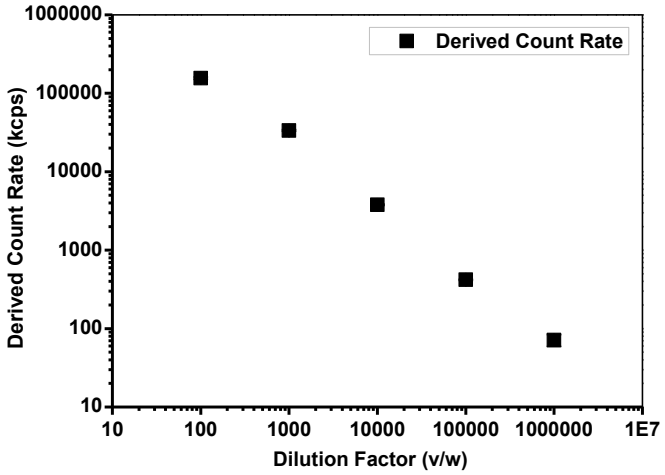


Figure 4-7. The relationship between Dilution Factor and Derived Count Rate for VPG release samples.

The particle properties in the release samples of the FTC with membrane model are shown in Figure 4-8 (A B C). The size (intensity) was increasing slightly up to day 34 and the DCR of 30% VPG was also increasing up to day 34, which indicates that erosion happened in that time.

With the membrane, the count rate was very low, and for 40% and 50% VPG almost no lipid goes out (Figure 4-3). For the 30% VPG groups, after about 30 days more lipid goes out. Consequently, the size and PDI data for 40% and 50% are not meaningful. For 30%, the data indicate a size of about 200-400 nm.

Without membrane, we have a much higher count rate. It looks like after 15 days, the 30% and 40% formulations are already eroded fully (Figure 4-4) and not much more is coming out.

Regarding Z-Ave/Size/PDI it is clear that no small, well defined liposomes are release but a very wide range of large liposomes.

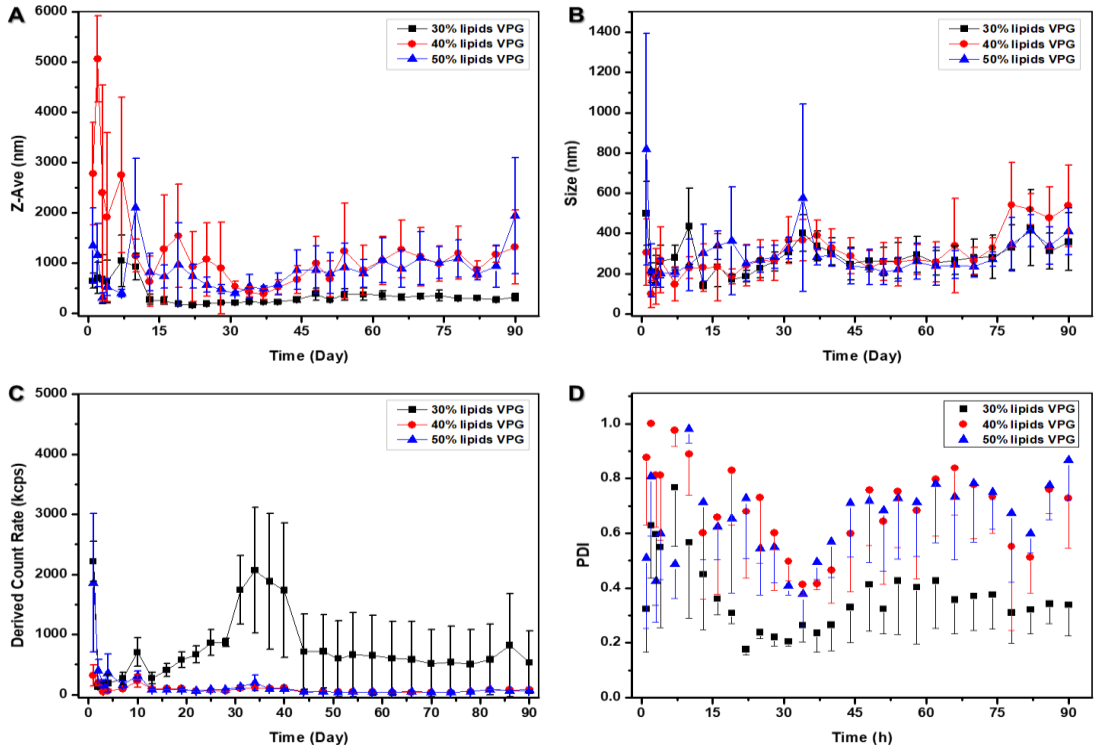


Figure 4-8 The DLS analysis of released fractions from FTC with membrane (5 µm pore size). (A) The Z-Ave size value of released fraction. (B) The Size of intensity value of released fraction. (C) The Derived Count Rate value of released fraction. (D) The PDI value of released fraction.

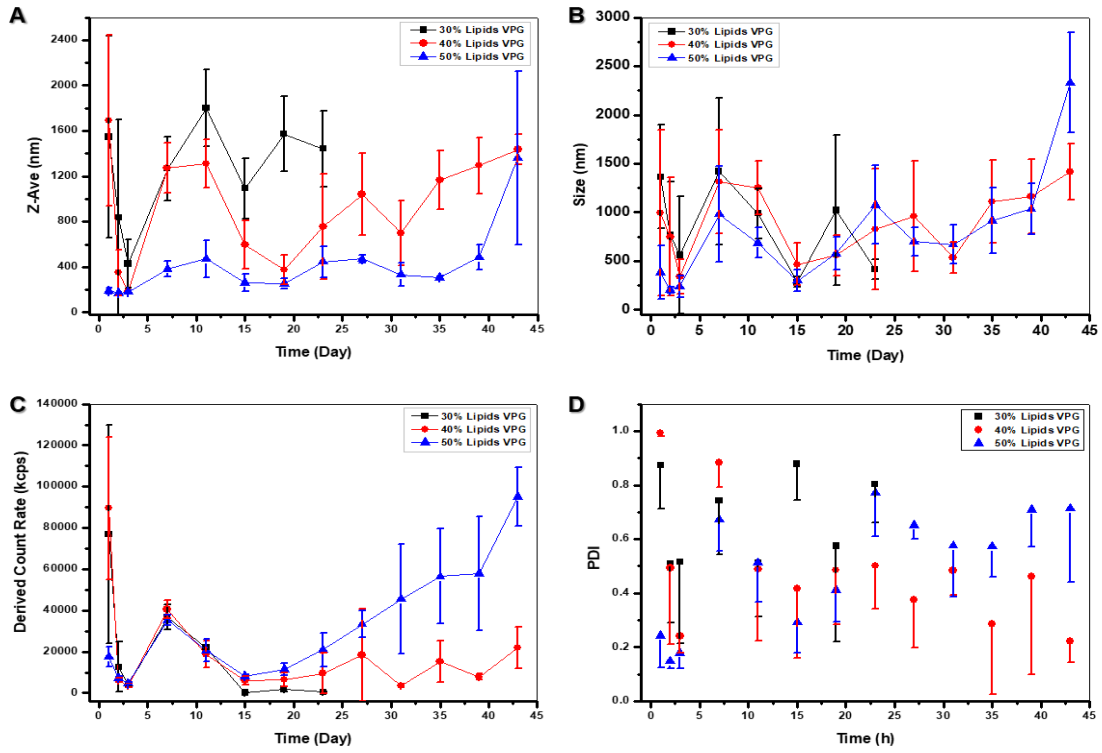


Figure 4-9 The DLS analysis of released fractions from FTC without membrane. (A) The Z-Ave size value of released fraction. (B) The Size of intensity value of released fraction. (C) The Derived Count Rate value of released fraction. (D) The PDI value of released fraction.

The released fractions from the FTC with membrane were analyzed by Nanoparticle Tracking Analysis (NTA) (Table 4-1). From the FTC with membrane model, the released

fractions from different VPG formulations at different time points were brought to Scanning Electronic Microscopy (SEM) for morphology analysis. At Day 7, the particles numbers were larger than at day 28 and 54 (Figure 4-10).

NTA				DLS			
Day 7	30% lipid VPG	40% lipid VPG	50% lipid VPG	Day 7	30% lipid VPG	40% lipid VPG	50% lipid VPG
Mean (nm)	346.22	377.11	269.44	Size. Int. (nm)	280.22	148.14	207.53
D90 (nm)	584.22	484.67	364.89	Z-Ave (nm)	1050.04	2754.11	399.18
D70 (nm)	401.56	424.44	301.67	PDI	0.768	0.977	0.489
particles / mL	9.79E+08	6.65E+08	1.40E+09	Derived Count Rate (kcps)	269.1	98.1	164.6
Day 28	30% lipid VPG	40% lipid VPG	50% lipid VPG	Day 28	30% lipid VPG	40% lipid VPG	50% lipid VPG
Mean (nm)	195.44	279.89	98.89	Size. Int. (nm)	260.59	264.85	283.32
D90 (nm)	306.00	435.44	154.11	Z-Ave (nm)	216.90	897.63	485.21
D70 (nm)	220.67	325.56	112.89	PDI	0.220	0.602	0.549
particles / mL	2.93E+09	3.33E+08	3.73E+08	Derived Count Rate (kcps)	866.8	63.8	88.4
Day 54	30% lipid VPG	40% lipid VPG	50% lipid VPG	Day 54	30% lipid VPG	40% lipid VPG	50% lipid VPG
Mean (nm)	184.67	210.22	197.89	Size. Int. (nm)	265.04	259.26	220.78
D90 (nm)	290.44	352.67	379.22	Z-Ave (nm)	377.39	1245.56	920.09
D70 (nm)	205.78	252.11	221.67	PDI	0.426	0.752	0.728
particles / mL	1.80E+09	2.11E+08	2.13E+08	Derived Count Rate (kcps)	663.3	44.8	45.4
Day 70	30% lipid VPG	40% lipid VPG	50% lipid VPG	Day 70	30% lipid VPG	40% lipid VPG	50% lipid VPG
Mean (nm)	198.44	272.56	203.22	Size. Int. (nm)	280.83	263.29	235.14
D90 (nm)	317.33	507.89	346.33	Z-Ave (nm)	344.66	1125.72	1102.84
D70 (nm)	218.44	329.22	236.11	PDI	0.371	0.776	0.784
particles / mL	2.07E+09	1.50E+08	1.90E+08	Derived Count Rate (kcps)	519.8	38.4	38.6

Table 4-1 The VPG released fraction analyzed by NTA and DLS of FTC with membrane

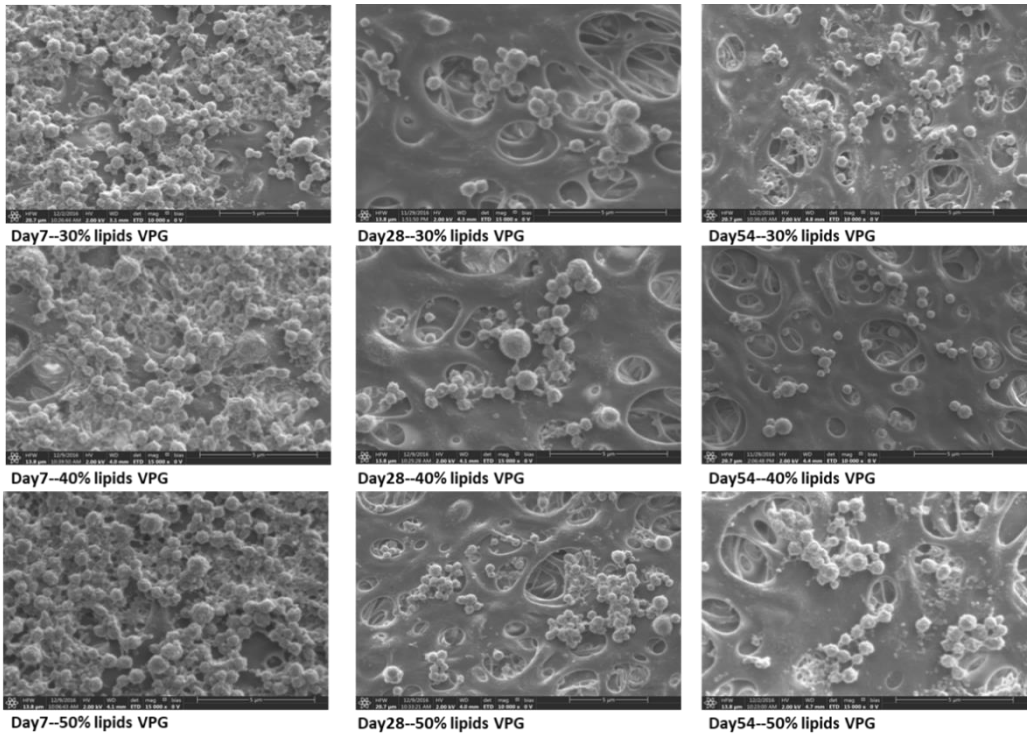


Figure 4-10 The released particle from VPG in FTC with membrane model by SEM (the bar is 5 μm). The background is the smooth surface of regenerated cellulose filter membrane.

Lower flow through rate (0.25mL/min) was tested also (Figure 4-11), for the FTC without membrane.

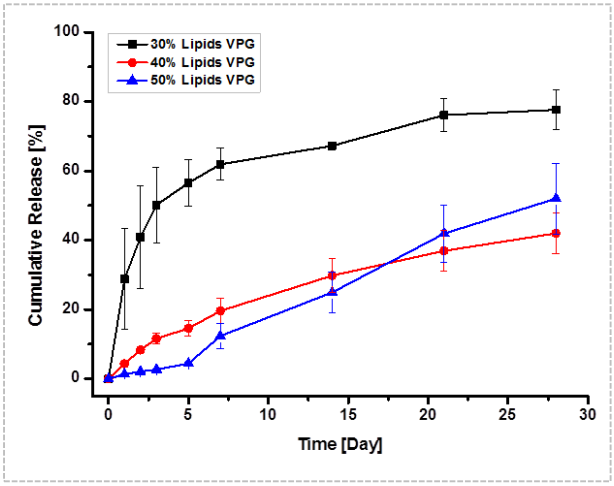


Figure 4-11 Cumulative release of FITC-Dextran (70K) from VPG in the flow-through release cell, which during the 0.25 mL/min flow-through rate. (FTC without membrane)

According to the measurements, the 30% lipids VPG group releases similar to 0.5 mL/min conditions, but the release behaviors of 40% and 50% lipids VPG groups were very close, which demonstrates that a lower flow rate is not a good setting for discrimination different VPG formulations erosion and release study. Therefore, this flow rate was not pursued in the further study.

4.2.3 The Agarose Gel (AGG) release test model for VPGs

4.2.3.1 The Quantitative Analysis of Agarose Gel (AGG) release test model for VPGs by sampling

The Agarose Gel (AGG) model is a visible release model, which can support the release into surroundings that mimic tissues. The main mechanism in AGG release model is diffusion, which is similar to the major penetration process into the blood vessels or lymph node after subcutaneous administration (Figure 4-12), when only a minor amount of liposomes is eroded and the drug diffuses in free form. The aqueous nature of the gel makes it practically impossible to allow relevant amounts of liposomes to diffuse out.

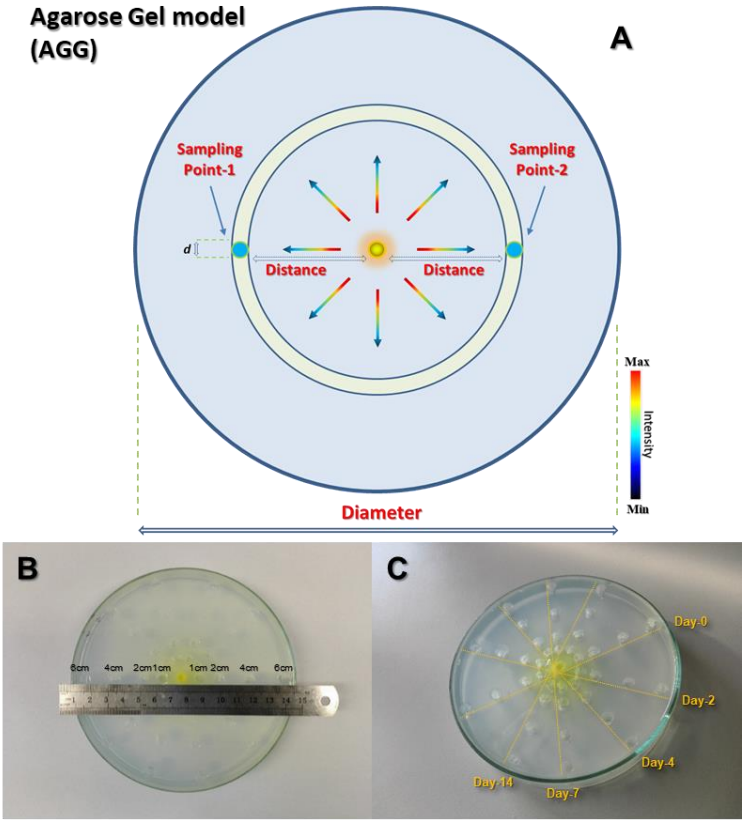


Figure 4-12 The Agarose Gel (AGG) model. (A) Schematic of AGG release test model, (B) (C) the AGG models after release sampling of five time points.

After the sampling from the AGG model (detailed method in Chapter 2), the sample aqueous solutions (Squeeze the liquid from gel samples) were measured quantitatively by Fluorescence Spectrophotometry, and then, the concentration at the special position point was calculated (all groups with triplicate) (Figure 4-13).

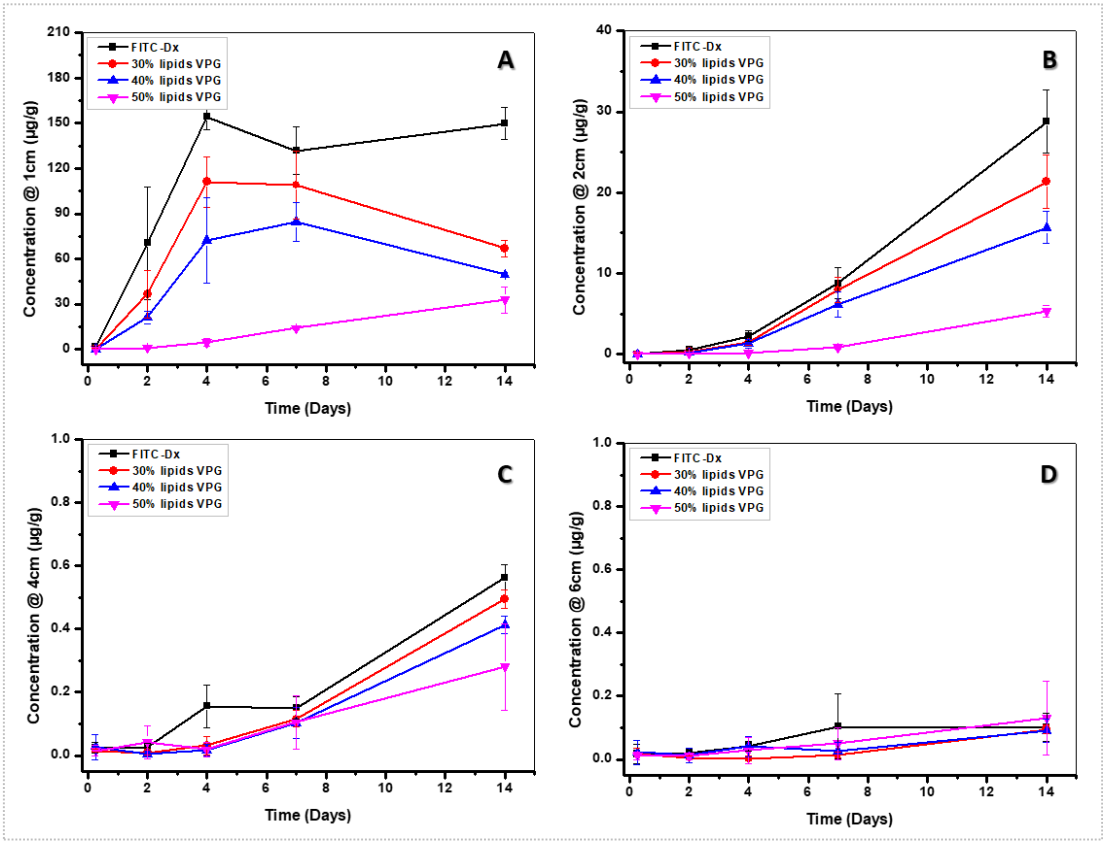


Figure 4-13 Quantitative Analysis of AGG release model in relevant locations. (A) The concentration trend in 1 cm distance from the central sample. (B) The concentration trend in 2 cm distance from the central sample. (C) The concentration trend in 4 cm distance from the central sample. (D) The concentration trend in 6 cm distance from the central sample (n=3).

At a distance of 1 cm from the center point (Figure 4-13 A), the concentration increased in all formulations during the first 4 day of the experiment. 30% and 40% lipid VPG formulations showed a slightly decreased release rate, and the 50% lipid VPG samples showed a sustained release during the whole release study period. At the other distances (2, 4, 6 cm) the concentrations reached were small, but increased steadily after 2 to 14 days.

Because the hydrophilic drug molecules diffusion in agarose gel is from the central point to the surrounding larger circle (from high concentration to low), which the 2-D FITC-Dextran distribution in the agarose (petri dish) is like the sunlight from the “sun” point (Figure 4-12, A). According to the measurement for FITC-Dextran concentration in a relevant position of the agarose gel, the concentration map for a total content of FITC-Dextran in agarose could be calculated. By this, the total cumulative release from VPG in relevant time points was got (Figure 4-14).

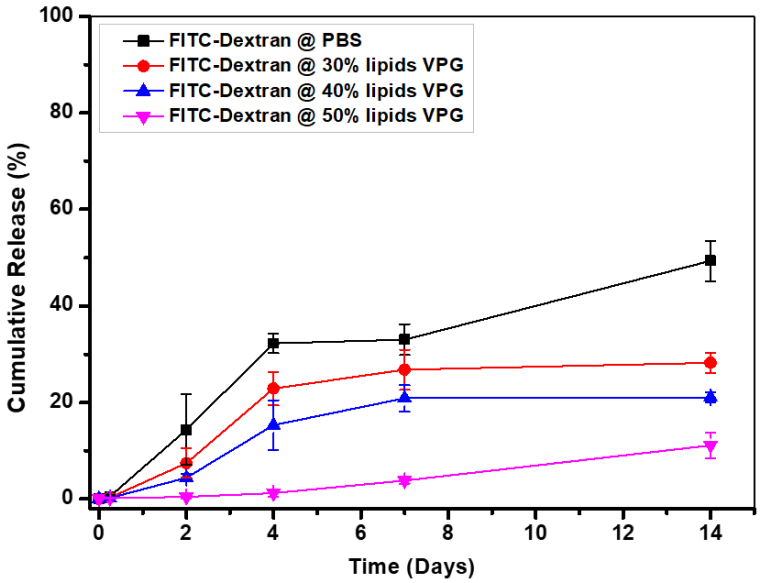


Figure 4-14 The cumulative release of FITC-Dextran from the VPGs in AGG release models

From the cumulative release results, the release behavior of different formulations is shown in Figure 4-14, and the release rate of the model drug (FITC-Dextran) was decreased as expected by increasing lipid concentrations, and the release model is well capable of describing different formulations. Surprising, the depot effect compared to the plain solution of the model composed is not very strong for 30% and 40% VPG in the first seven days. It appears that the diffusion of the dextran itself is very slow.

4.2.3.2 The Semi-Quantification Analysis of Agarose Gel (AGG) release test model for VPGs by imaging

At the same time, the AGG model could be used for direct imaging of the drug diffusion in the gel. The FITC-Dextran was traced by the fluorescence imaging system, which offered another approach to analyze the release behavior of VPG formulation easier and faster. Different lipid content VPG formulations were prepared and the FITC-Dextran was added as the model drug, and then the AGG dishes (with samples) were brought to the incubator and imaging at different time points (all groups with triplicate) was carried out. The FITC-Dextran loaded VPG release behaviors were obtained and are shown in Figure 4-15. The release rate of FITC-Dextran was decreased by increasing lipid concentrations.

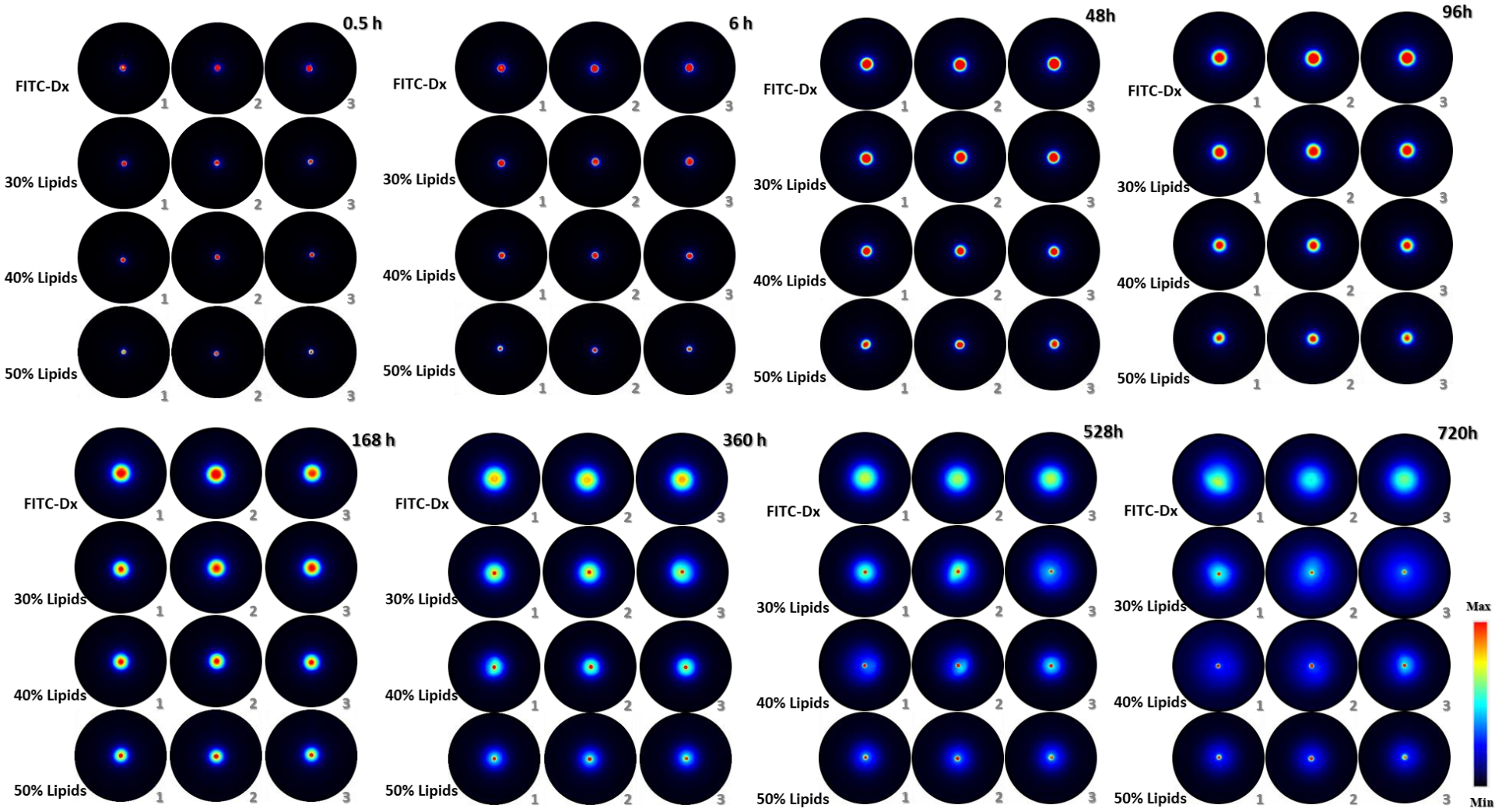


Figure 4-15 The fluorescence imaging of FITC-dextran loaded VPGs in AGG models in a series of time points.

Based on the intensity of the fluorescence signal, the semi-quantification measurement was performed using gray model graphs (Figure 4-16). With the treatment by imageJ® software, the mean intensity value in special distance cycles was obtained, and then the cumulative release status could be calculated (Figure 4-17).

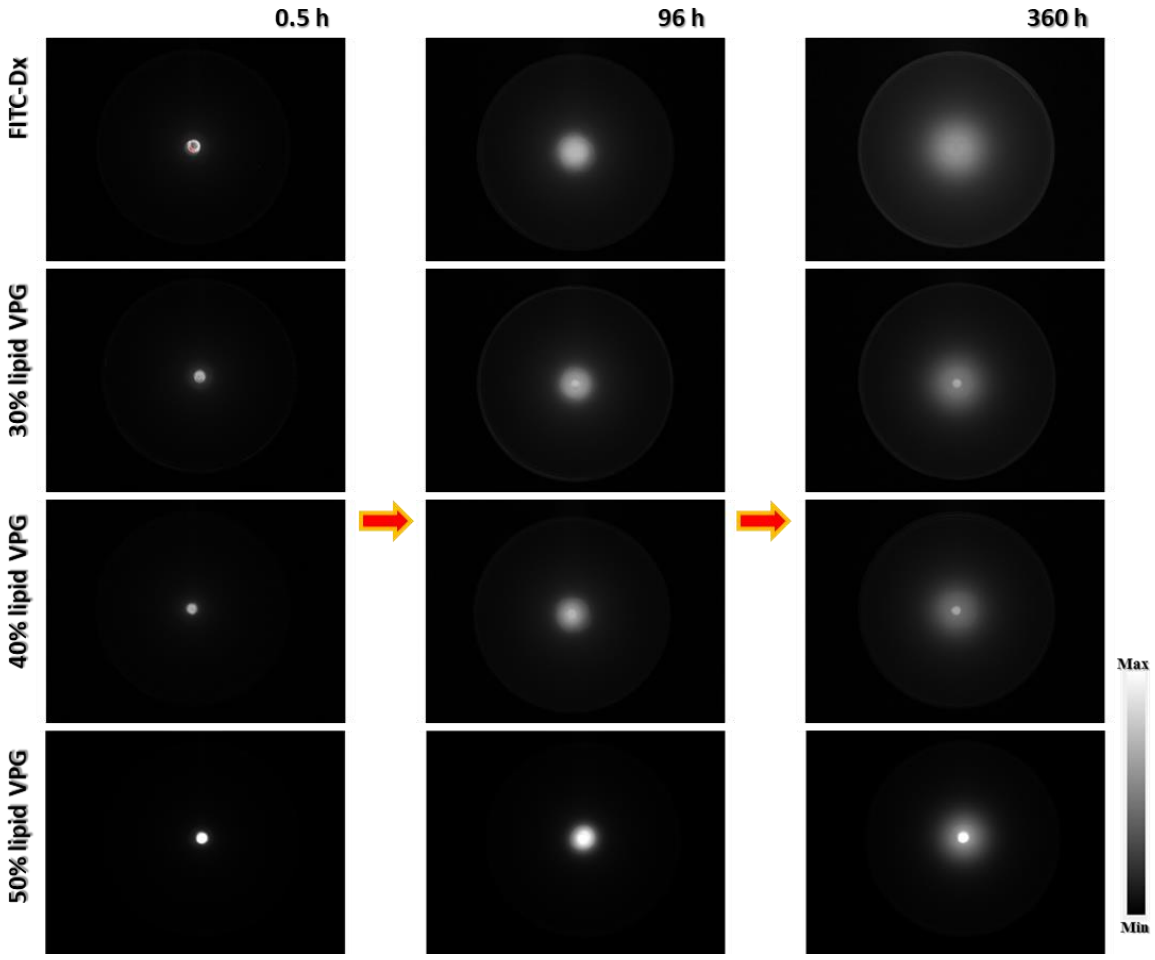


Figure 4-16 The gray model graphs of FITC-dextran loaded VPGs in AGG models from fluorescence imaging in main time points.

The FITC-Dextran diffused into the surrounding agarose gel from the VPG in the donor chamber, and the surrounding intensity value of fluorescence was measured at special time points, and the signal value was calculated as the marker of FITC-Dextran in the release, and the intensity of fluorescence signal was described by arbitrary units (a.u.) here. The basic release capability of different VPG formulations was demonstrated. This method is an easy way to get release results of long-term release formulations quickly (Figure 4-17).

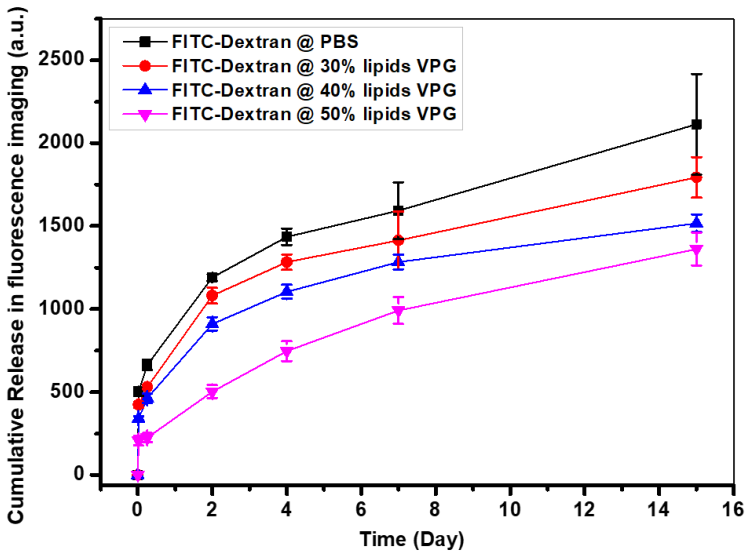


Figure 4-17 The semi-quantification measurement of FITC-Dextran loaded VPGs release behavior by the fluorescence intensity in imaging (15 day).

The AGG release model supports two options for the VPG release investigation. Comparing the semi-quantification measurement (by imaging, Figure 4-17) with the quantification measurement (by sampling, Figure 4-14), the general trends about the release behaviors between those formulations were similar. The higher the lipid content in VPG is the slower release will the behaviors be, and both of the two methods could separate the release behaviors of 30%, 40%, and 50% lipid VPGs.

The imaging method supports an easy and non-destructive way to measure the release process, and the measurements could be done with lots of time points and over a more extended measurement period (Figure 4-15, for 30 days). The disadvantages are that the signal (Fluorescence/UV/Infrared/Raman) of drug molecules must have enough intensity for detection. The quantification measurement with the sampling method (Figure 4-14) could support more accurate results for most types of drug molecules, but needs a series of handling steps. The disadvantage of the sampling methods is that the sampling time is limited because of limited gel surface area could not support enough positions for more measurements.

4.2.4 The small filter holder (SFH) release test model for VPGs

The small filter holder is a common tool in laboratory. In this part the small filter holder was modified for release cells (Figure 4-18). During the model preparation, the female

Luer-Lok fitting was removed and closed with a plastic piece to build the donor chamber for VPG sample, at the same time, the output terminal fitting was cut off as the release window (⌀: 0.95 cm).

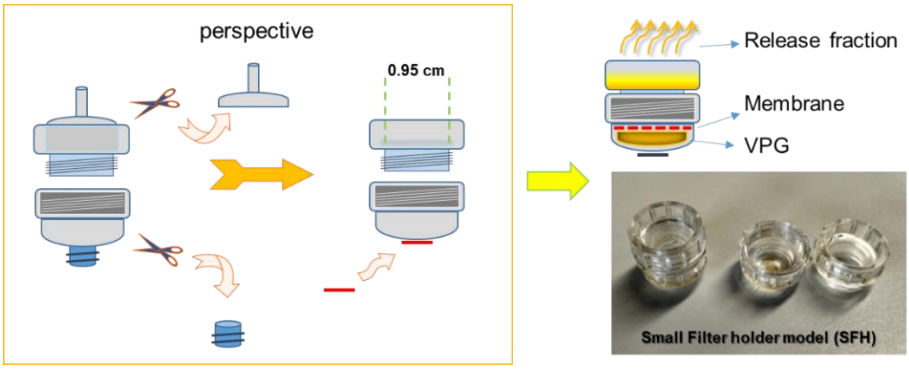


Figure 4-18 schematic of small filter holder (SFH) release model

The 5 µm pore size membrane was brought into the SFH model, and a solution of FITC-Dextran in 20 mM PBS was placed inside of the SFH model and the released was measured (Figure 4-19).

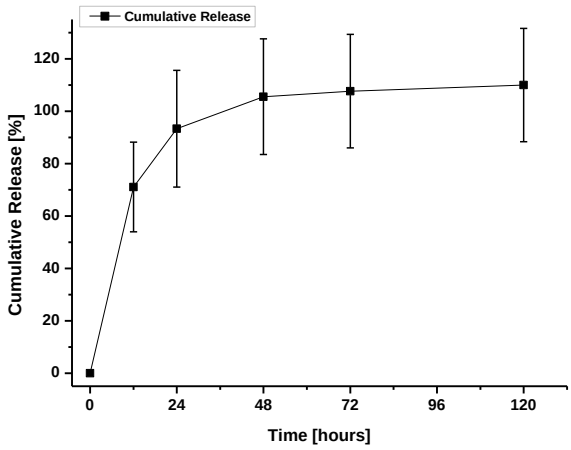


Figure 4-19 FITC-Dextran aqueous solution release behavior in Small filter holder.

The results demonstrated the FITC-Dextran was released completely in about 24 hours similarly to the flow through cell (FTC) with membrane model, which means the model could work in VPG formulation investigation.

The FITC-Dextran loaded VPG formulations were tested in the SFH model (with **5 µm** pore size membrane). The FITC-Dextran and lipid release behaviors of high lipid content VPG formulations were very close (figure 4-20, A, B), and the model could not differentiate high lipid content samples (40%, 45%, and 50% VPG). On the other hand,

the lipid release (Figure 4-20 B) was higher than FTC model with same membrane (Figure 4-3 B), which means more lipid particle were released in the incubation shaking progress. At the same time, the SFH model with **80 µm** pore size membrane was also tested. According to the figure 4-20 C, D, during this group, the release situation of 30% and 40 % were close.

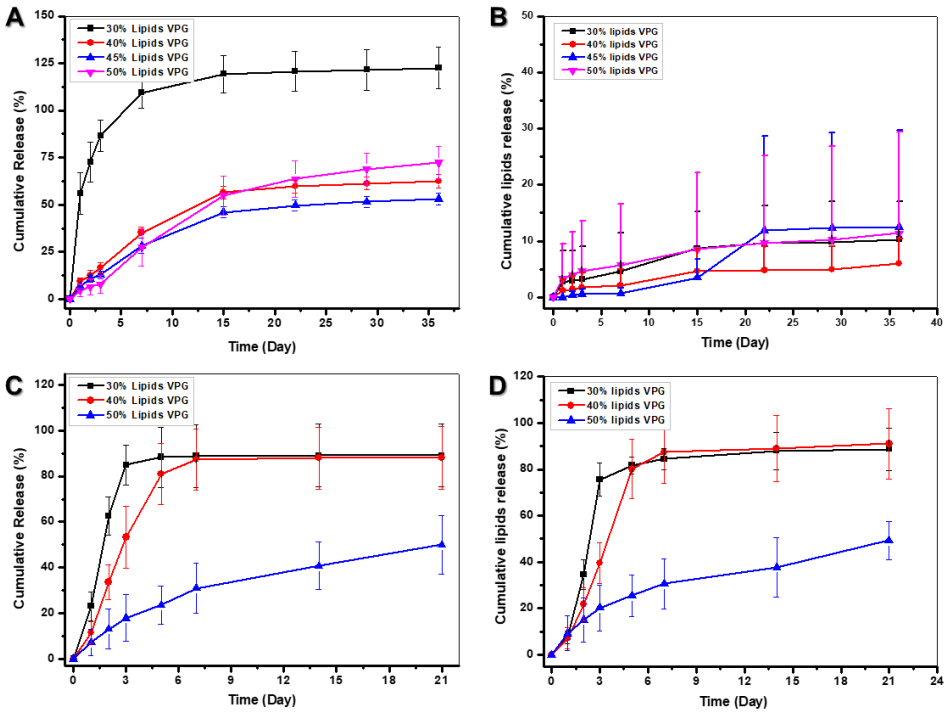


Figure 4-20 The SFH release behavior with different pore size membranes. (A) FITC-Dextran and (B) lipid release behaviors in SFH with 5 µm pore size membrane. (C) FITC-Dextran and (B) lipid release behaviors in SFH with 80 µm pore size membrane.

Finally, after the previous experiments, a **40 µm** pore size membrane was added to the SFH models, and the release behavior can be differentiated in this system. The 30%, 40% and 50% VPG formulations showed different release rates (figure 4-21). FITC-Dextran and lipid release behaviors were very close, which means they were released together, but with a certain shift or delay (Figure 4-21, C, D, E).

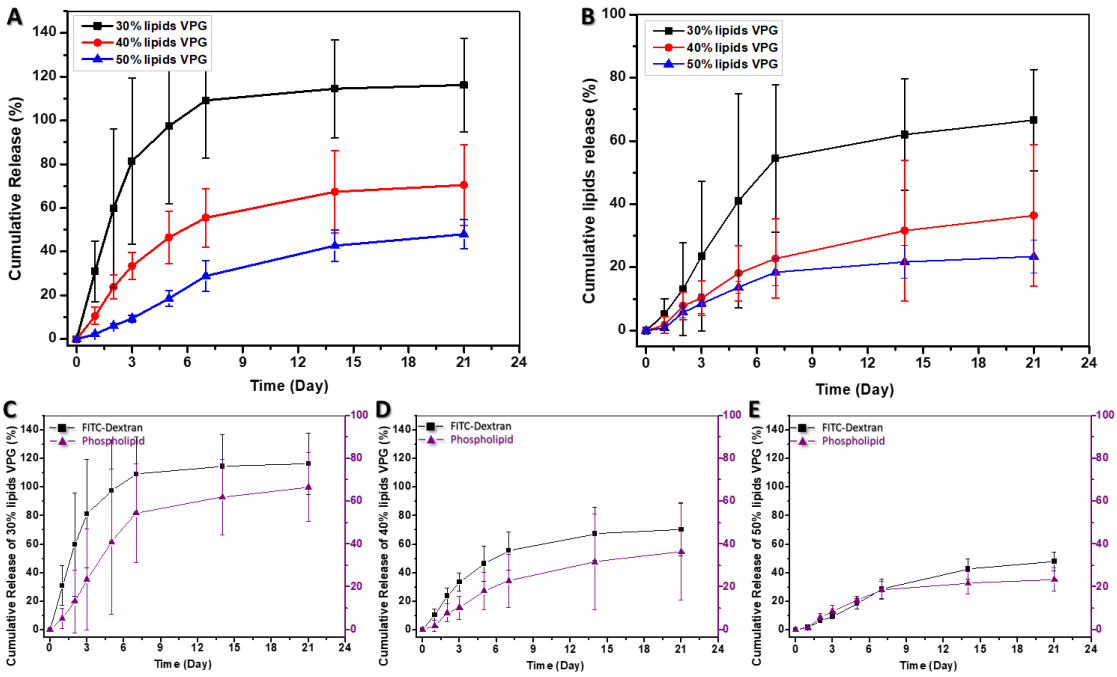


Figure 4-21 The SFH release behavior with different 40 μm pore size membranes

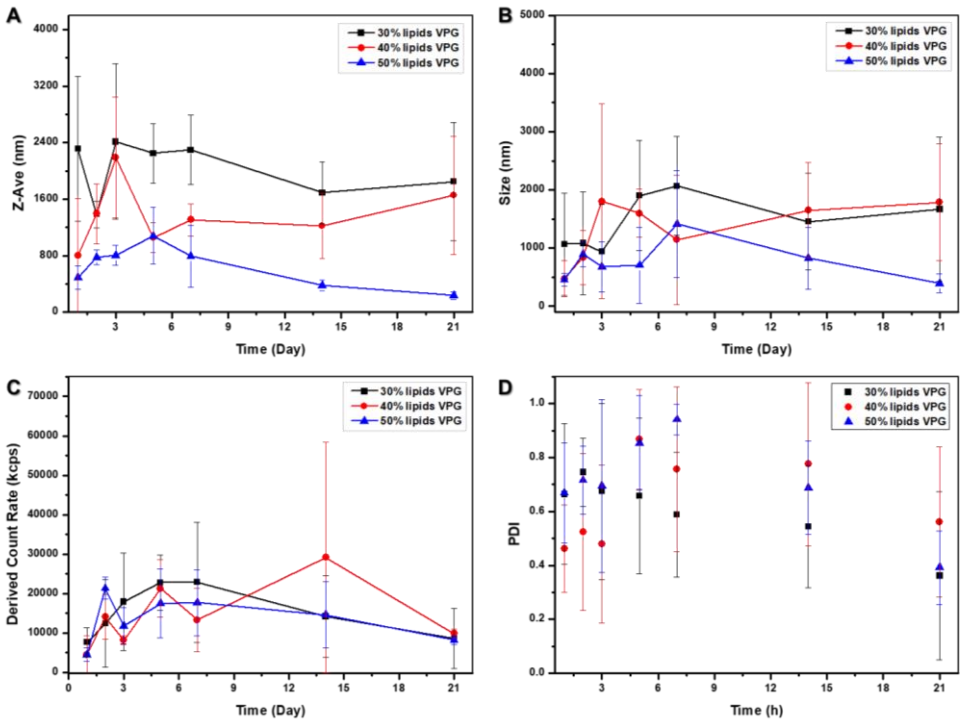


Figure 4-22 The DLS analysis of fraction release behavior in SFH with 40 μm pore size membranes. (A) The Z-Ave size value of released fraction. (B) The Size of intensity value of released fraction. (C) The Derived Count Rate value of released fraction. (D) The PDI value of released fraction.

The released fraction was also analyzed by DLS (Figure 4-22), the z-average values of particle size was larger than the ones obtained from the FTC with membrane release model. The release peak (DCR) of 30% and 40% lipid VPG formulations appeared on day 7, and the release out of the high lipid content formulation (50% VPG) was slowed down and the release peak appeared on day14.

After the release experiments, the SFH release cells were placed as shown in Figure 4-23. For the 30% lipid VPG formulations the samples nearly released completely, while for the 50% lipid VPG formulation the drug and lipid were still kept in the holders.

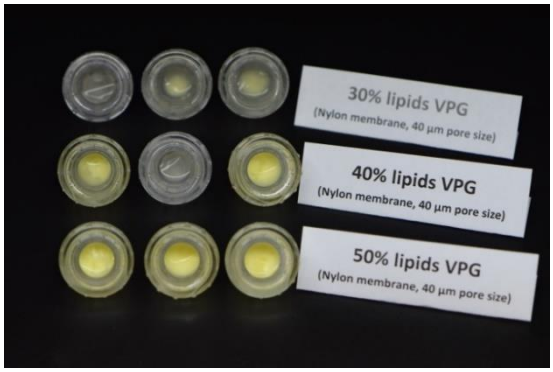


Figure 4-23 The final statue of VPG release experiment in SFH with (40 µm pore size) membrane

In summary, the 40 µm pore size membrane could differentiate the release out of different lipid content VPG formulations. It was feasible for VPG release fast and easy studies.

4.2.5 Summary evaluation of different *in vitro* release test models for macromolecular drug loaded VPG formulations

Four different *in vitro* release test models have been assessed developed in this research, and they show different performances for the investigation of VPG formulations. According to the release results of all groups, the FITC-Dextran release data of the same lipid content VPG formulations were put together to compare different release test models (Figure 4-24). For each lipid content VPG formulation, the release in FTC with membrane groups was slowest. The release rate in FTC model without membrane was fastest for 30% and 40% lipid VPG formulations, and the SFH model was fastest for the 50% lipid VPG formulations.

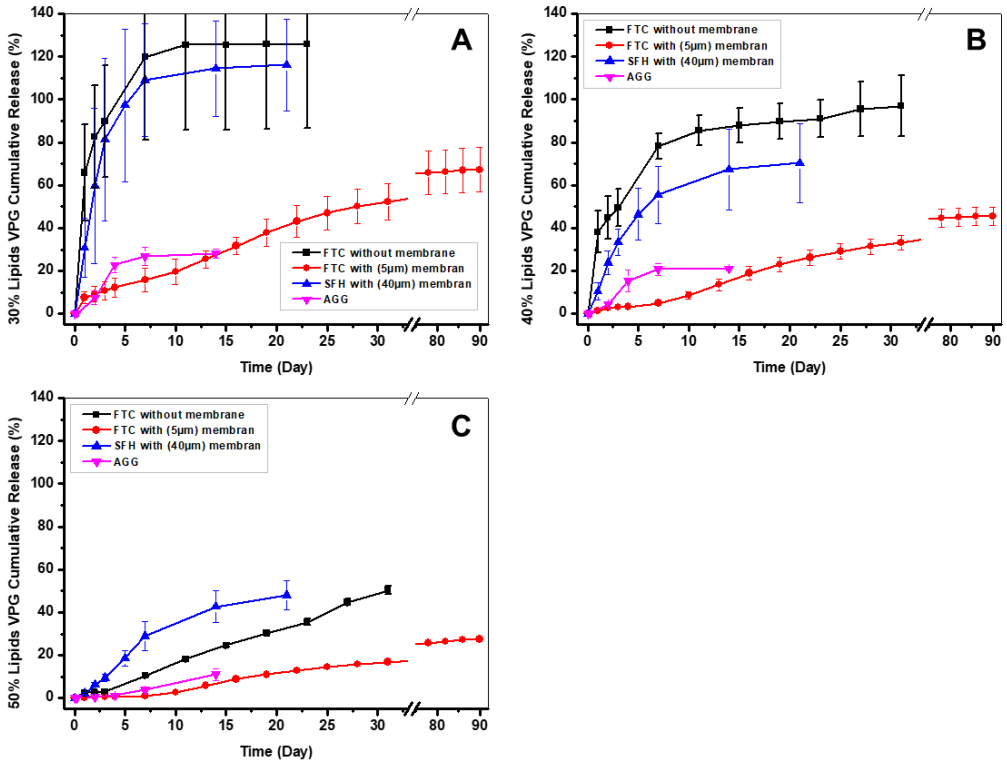


Figure 4-24 The summary of FITC-dextran release in different release models. (A) The release behaviors of 30% lipids VPG in different release models. (B) The release behaviors of 40% lipids VPG in different release models. (C) The release behaviors of 50% lipids VPG in different release models.

The studied models follow a different path to mimic the tissue situation for the VPG after subcutaneous administration, and thereby support different aspects to evaluate the VPG formulations (Table 4-2).

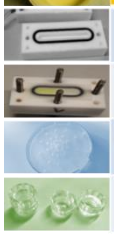
	Test Model	The release rate of free drug	The release rate of lipid particles	Multi-channels test Capability	Advantage during VPG release research
	Flow-through Model without membrane (FTC w/o membrane)	Fast	Fast	Low	Lots of previous cases
	Flow-through Model with membrane (FTC with membrane)	Slow (depend on the membrane)	Very Slow (depend on the membrane)	Low	Release mechanism (free drug vs. liposomes)
	Agar gel model (AGG)	Slow	Very Slow	Quantitative analysis: Low. Imaging analysis: Medium	Visualization
	Small filter model (SFH)	Medium	Medium	High	Easy operation and good resolution

Table 4-2 The general comparison of different erosion and release test model.

The advantage of the FTC with membrane model lies in the good capability to investigate the release behaviours precisely in detail, and to mimic the *in vivo* tissue situation for drug adsorption by blood or lymph capillaries. But both the FTC with or w/o membrane methods need lots of equipment, the test channels are expensive, and it is necessary to refill the release medium every two or three days.

The AGG model is a visible model, which can differentiate different VPG formulations directly, but the main force of drug release is diffusion, the release rate was small, and it could not show the erosion status of the semi-solid formulation.

The SFH model was a new model for VPG formulation development, and it offered an easy and fast way for investigation of the release behavior of VPG formulations. And it allows to include the erosion from the VPG local depot matrix in a rather controlled manner, by using a coarse screen filter. Compared to the FTC without membrane, the erosion process is better controlled despite a much simpler and cheaper experimental setup.

4.3 Erosion and release study of small molecule loaded VPGs

SAFit2 is a hydrophobic small molecule, which has a strong capability as a novel FKBP51 antagonist and is studied as an experiment drug right now. According to previous research, the $t_{1/2}$ of SAFit2 (in aqueous solution) was about 9.7h (during the subcutaneous injection in 10 to 12-week old male C57BL/6 mice), and VPGs offered a sustained release platform for SAFit2 for the treatment of diseases [15]. SAFit2 loaded VPGs were prepared as depot formulations, and the *in vitro* release behavior and in vivo PK performance were investigated (Figure 4-25).

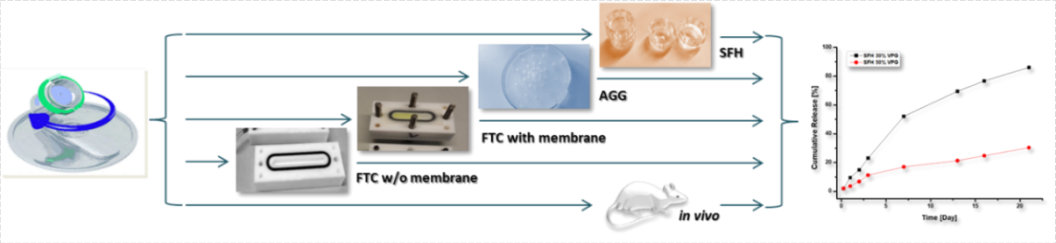


Figure 4-25 Schematic overview of *in vitro* and *in vivo* study correlation *in vitro* release with PK parameters.

4.3.1 *In vitro* release test study

SAFit2 was encapsulated in 30% and 50% VPGs, with a final concentration of SAFit2 of 10 mg/g gel. The SAFit2 loaded VPGs were placed into the *in vitro* release models for the release study; the sampling time points are given in Table 4-3.

SAFit2@VPG *in vitro* release study calendar												
In vitro model	Setting	Time point (D=day)										
Flow Through Cell(FTC)	without membrane	**6 hours**	**12 hours**	**D1**	**D2**	**D3**	**D4**	**D7**	**D10**	**D13**	**D16**	**D21**
	with membrane (5 μm)	**6 hours**	**12 hours**	**D1**	**D2**	**D3**	**D4**	**D7**	**D10**	**D13**	**D16**	**D21**
Small Filter Holder(SFH)	with membrane (40 μm)	**6 hours**	-	**D1**	**D2**	**D3**	-	**D7**	-	**D13**	**D16**	**D21**
Agarose Gel	0.5% (w/v)	**6 hours**		**D1**	**D2**		**D4**	**D7**			**D16**	**D21**

Table 4-3 SAFit2@VPG *in vitro* release study: predetermined sampling time points.

Because the SAFit2 is a hydrophobic small molecule, pH 7.4, 20 mM PBS with 4% (m/v) bovine serum albumin (BSA) was used as release buffer for FTC (with & w/o membrane) and SFH release models. These experiments were completed together with Michaela Breitsamer (Pharm. Technology and Biopharmaceutics, LMU, Munich). After *in vitro* release experiments, these released fractions were analyzed by RP-HPLC. At the same

time, the phospholipid release from the VPG formulation was determined by the Phospholipid assay kit (Wako, Lab Assay). The SAFit2 and lipid release were analyzed for the comparison between different *in vitro* release test models. The SAFit2 concentrations in the FTC with membrane and AGG model were lower than the detection limit, which means the release of SAFit2 in these models was very slow and limited. The samples obtained from the SFH model and the FTC model without membrane showed the following release behavior (Figure 4-26).

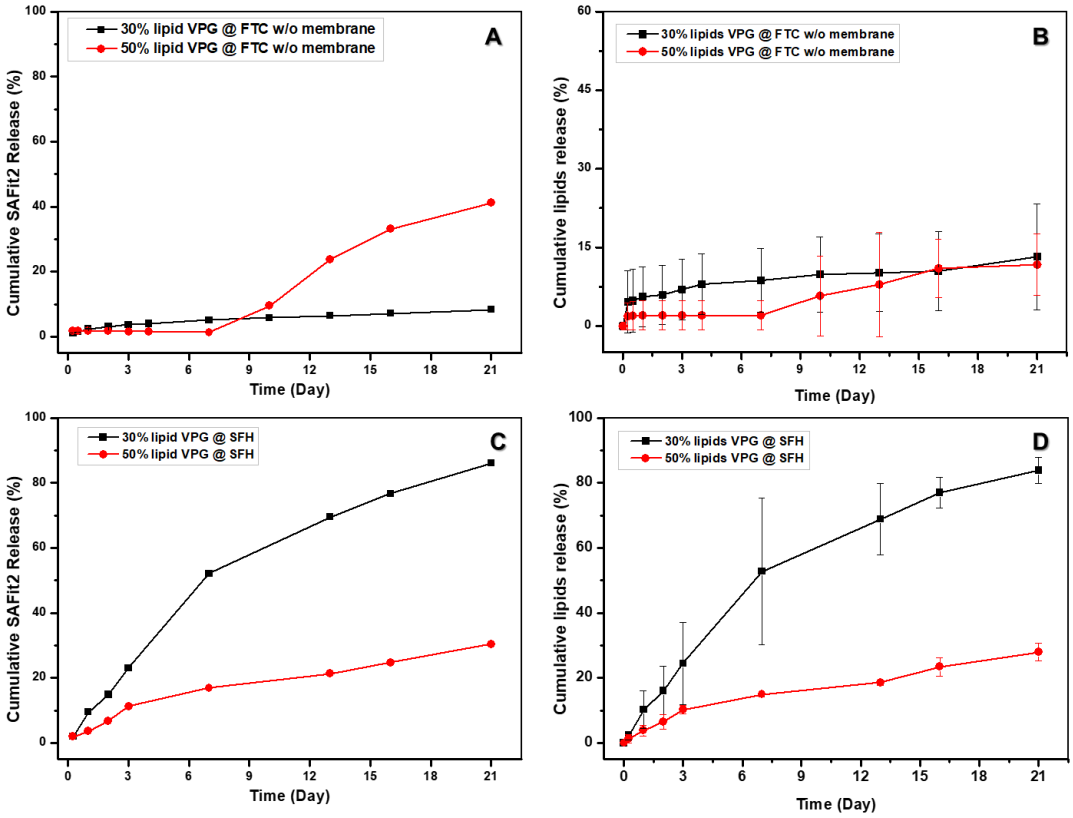


Figure 4-26: The release behaviors of SAFit2 loaded VPG. (A) Cumulative release of SAFit2 in FTC release model without membrane. (B) Cumulative release of lipids from VPG in FTC release model without membrane. (C) Cumulative release of SAFit2 in SFH, (D) Cumulative release of lipids from VPG in SFH.

As can be seen in figure 4-26, SAFit2 showed a very slow release rate for the 30% and 50% VPG formulation in the FTC w/o membrane model in the first 7 days, but then the release rate of the 50% lipid VPG formulation increased significantly. The SFH model differentiated the formulations better. The release of the 30% lipid VPG formulation increased faster than the one of the 50% VPG formulation. At the same time, the lipid and SAFit2 releasing were synchronized in the SFH model. From the *in vitro* release tests, the SFH showed good performance for the VPG *in vitro* release investigation.

4.3.2 *in vivo* Pharmacokinetic (PK) experiment

The SAFit2 loaded VPG formulations (with 30% and 50% lipids) are tested *in vivo* after subcutaneous (SC) administration in mice (C57Bl/6N, n=7 & 8). As a control SAFit2 in aqueous solution (20% EtOH, 40% propyleneglycol, 5% Tween 80, 5% PEG 400 in 0,9% saline) was also tested as subcutaneous administration. All formulations were injected subcutaneously with a dose of 10 mg/kg body weight. The *in vivo* party study, which included the animal breeding, administration, blood samples taken and bio-samples LC-MS/MS analysis was done by the Max-Planck-Institute of Psychiatry (Munich, Germany). In the PK study, the blood samples were taken at pre-determined time-points (Table 4-5).

SAFit2@VPG *in vivo* release study calendar											
Group Setting	Time point (D=day)										
SAFit2 in buffer	**0.5 hours**	**2 hours**	**6 hours**	**12 hours**	**D1**	**D1.5**	**D3**	**D4**			
SAFit2 @ 30% VPG	---	---	**6 hours**	**12 hours**	**D1**	**D1.5**	**D3**	**D4**	**D7**	**D10**	**D13**
SAFit2 @ 50% VPG	---	---	**6 hours**	**12 hours**	**D1**	**D1.5**	**D3**	**D4**	**D7**	**D10**	**D13**

Table 4-5 The sampling calendar of in vivo PK study

After measurement and calculation, the plasma concentration was obtained (Table 4-6, Figure 4-27). The blood concentration of SAFit2-solution shows fast release with a burst phase in the first 2 hours, and then slowly decreasing plasma levels. Both 30% and 50% VPG formulations showed sustained release for SAFit2.

Time	**Average value of SAFit2 in Plasma Concentration**		
	In Solution	**30% lipid VPG**	**50% lipid VPG**
day	**ng/mL**	**ng/mL**	**ng/mL**
0	0	0	0
0.02083	5374.27	--	--
0.08333	4273.39	--	--
0.25	3596.34	1601.35	1110.00
0.5	2804.41	1370.60	1211.02
1	1903.94	1148.04	1340.36
2	1459.63	758.53	1068.98
3	884.95	864.49	996.49
4	404.37	655.14	801.33
7	--	768.36	1042.95
10	--	577.48	787.48
13	--	452.82	541.27

Table 4-6 SAFit2 plasma concentration *in vivo* of different formulations in mice (C57Bl/6N).

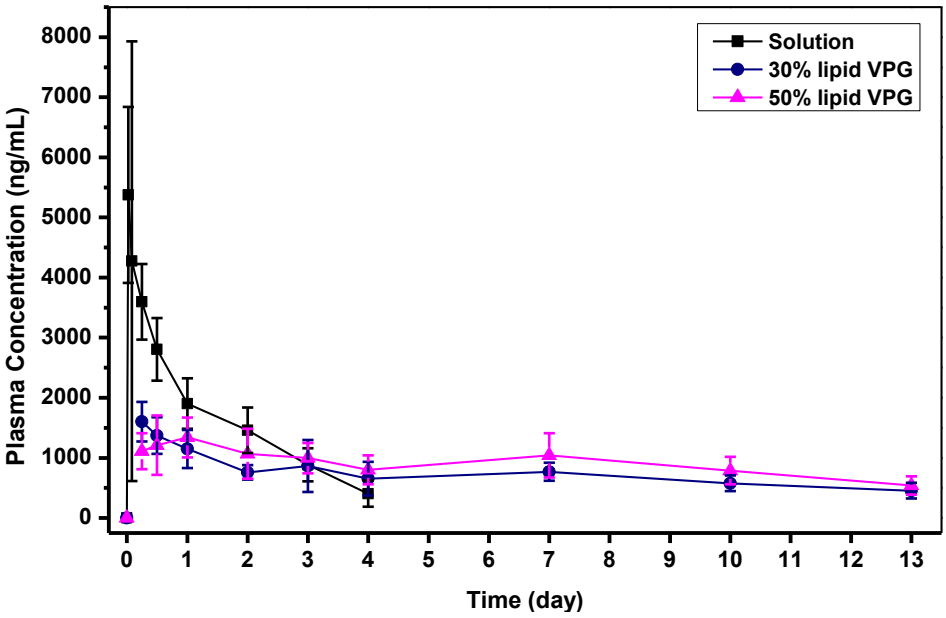


Figure 4-27: SAFit2 plasma concentration *in vivo* of different formulations in mice (C57Bl/6N).

The pharmacokinetic parameters were calculated based on the plasma concentration by Dr. Jilong Wang and Prof. XiaoJiao Du (Institutes for Life Sciences and School of Medicine, South China University of Technology, Guangzhou, China) using a non-compartmental model in the Data Analysis System 3.2.6 pharmaceutical in vivo calculation software; BioGuider (co., Shanghai). Relative bioavailability was calculated with the following equation.

$$F_{\mathrm{rel}} = 100 \cdot \frac{AUC_{\mathrm{A}} \cdot D_{\mathrm{B}}}{AUC_{\mathrm{B}} \cdot D_{\mathrm{A}}}$$

The basic pharmacokinetic parameters were calculated as follows (table 4-7).

	Solution	**30%lipids VPG**	**50%lipids VPG**
$t_{1/2}z$ (h)	28.3	209.6	229.4
T_{max} (h)	2	7.2	38
C_{max} (ug/L)	20	6.2	5.92
AUC(0-t) (ug/L*h)	546.8	699.6	1027.8
F_{rel}(0-t) (%)	--	127.9	188
AUC(0-∞) (ug/L*h)	617.4	1224.7	1850
F_{rel}(0-∞) (%)	--	198.3	299.6
CLz/F (L/h/kg)	17.9	9	7.2

Table 4-7, The Pharmacokinetic parameters of SAFit2 *in vivo* after subcutaneous administration of SAFit2 in solution and SAFit2 loaded VPG formulations in mice (C57Bl/6N).

Compared to the aqueous solution, the VPGs support sustained release for SAFit2 during chronic disease treatment, and $t_{1/2}$ of SAFit2 in VPG formulation formulations

was increased significantly. At the same time, the AUC and T_{max} were increased with increasing phospholipid concentration in the formulations, and meanwhile, the C_{max} values decreased significantly. This demonstrated that the SAFit2 release from 30% lipid VPG and 50% lipid VPG formulations showed a significant depot effect compared to the solution.

4.3.3 Summary of erosion and release studies of small molecule loaded VPGs

In these studies, the SAFit2 loaded VPGs were tested in both *in vitro* release measurements and in an *in vivo* PK study. In the PK study, the 30% lipid VPG has a faster release rate than the 50% lipid VPG, and the drug-blood concentration of 50% lipid VPG group increased until 24 h, and then constant release at least on this period. According to these *in vivo* results, the FTC model was not correct, as it shows did not a relevant release for 30% or 50% in the whole test period. The SFH model performed better to mimic the *in vivo* situation, and it could distinguish the different lipid content VPG formulations. Although the *in vitro* release rate in SFH still cannot fit *in vivo* completely, after a short burst release at the beginning, all groups have a slow release over days, leading to an elevated blood level for both 30% and 50% VPGs. The *in vitro* data at SFH indicate a biphasic release behavior, and the release behavior is more flat release curve. Even not perfect mimic, the SFH model performance as a cheap, easy, and accurate model for *in vitro* VPG release investigations.

4.4 Conclusion

Within this chapter, four different *in vitro* release models have been developed and evaluated for VPG formulations. These release models have presented different properties with advantages and disadvantages for *in vitro* investigation of VPG. The sustained release of FITC-Dextran loaded VPGs was up to 3 months in the FTC with membrane model, and the main release force is diffusion. The release test with FTC w/o membrane model was fast, and lots of large particle fractions were also released from formulations, which would not happen *in vivo*. The AGG model allows both a visible qualitative release analysis and an accurate quantitative measurement by applying imaging technology, which both can be useful for semi-solid or implant formulation studies. Because the diffusion was the main force during the AGG model release testing, the release of slow erosion of liposomes cannot be measured and it therefore appears to be a model that is not appropriate for VPG studies. In the SFH model release study, the release terms were about 3 to 6 weeks, and it was an easy and fast way to test the release behavior of VPG formulations.

The small hydrophobic molecular drug (SAFit2) loaded VPGs were tested by these models *in vitro*, and compared it with the in vivo PK study. In the FTC w/o membrane model and SFH model measurement, the SAFit2 and lipid could be detected in the VPGs' released fraction. The SFH could distinguish the different lipid content VPG formulations by different release behaviors, and the FTC models could not separate the release difference between VPGs. The SAFit2 loaded VPG formulations were tested in an *in vivo* PK study, and the relative bioavailability increased with increasing lipid concentration. The VPG formulations demonstrated to have a sustained release capability. Because SAFit2 is a hydrophobic drug, the diffusion during the release was very limited, and the FTC with membrane model did not work for the SAFit2 loaded VPG study.

4.5 Reference

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Chapter 5 Storage stability of monoclonal antibody loaded VPG systems

5.1 Introduction

VPGs are semi-solid, highly concentrated lipid formulations, which form a good vehicle for biomolecule drug delivery with very good sustained release performance. Although there are a lot of advantages for VPG formulations, there are still challenges for clinical application, and one of them is the restricted long-term storage. Because protein drugs and the vesicular gel lack long-term stability, the protein loaded VPG formulation should be used shortly after preparation (about two weeks after preparation). In this research, we developed a long-term stable VPG formulation for monoclonal antibodies (mAb) delivery.

Several approaches were tested to develop a long-term stable VPG formulation. Lyophilization as an industrial application dates from the time of World War II when the demand for blood plasma increased [1], and nowadays it is a unit operation commonly employed as a drying technology in the food and pharmaceutical industry [2]. Because the rate of most chemical and physical degradation reactions is decreased dramatically in the glassy state after freeze-drying [1-3], the lyophilized samples could be stored and transported well. Alternatively, the frozen state is another way to store samples with the advantage of easy operation in scientific research, and this is also the standard procedure for protein bulk storage in the pharmaceutical industry[4]. Both methods were tested for mAb loaded VPG formulations, and the long-term storage experiments were performed based on these two methods.

The long-term storage stability analysis of mAb loaded VPGs includes gel appearance stability, protein stability, lipid stability, and further stability indicating methods. The chemical and physical stability as well as the bio-affinity of the respective mAb were analyzed. The peroxidation of lipid was also analyzed as a stability indicating method for the lipid stability in lyophilized VPG samples. The release behavior is an important parameter for VPG formulations, and protein and lipid release were measured separately in the long-term storage study.

5.2 Freeze-drying of VPGs loaded with monoclonal antibodies: preparation and stability study

5.2.1 Freeze-drying method development for mAb loaded VPGs storage

Lyophilization is a powerful method to protect protein drugs during the long-term storage in biopharmaceutical research and development[5]. During this part, the lyophilization method was brought into VPG storage study, first the mAb loaded VPGs were prepared by DAC (3500rpm, 45min, 2~8°C) as previous research. Then the VPG samples were treated with freeze-dryer for lyophilization. Finally, after storage, the dry VPG was reconstituted by magnetic stirring method before using, and the general procedure for VPG freeze-drying and reconstitution was placed as follows (Figure 5-1).

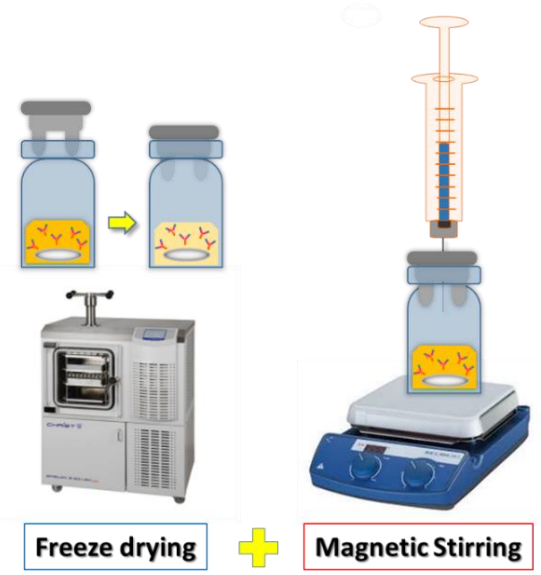

Figure 5-1 Scheme of mAb loaded VPG formulation storage by freeze-drying and reconstitution with magnetic stirring method.

5.2.1.1 The freeze-drying for VPG formulations

VPGs with different lipid concentrations were lyophilized and subsequently reconstituted by magnetic stirring (Figure 5-2).

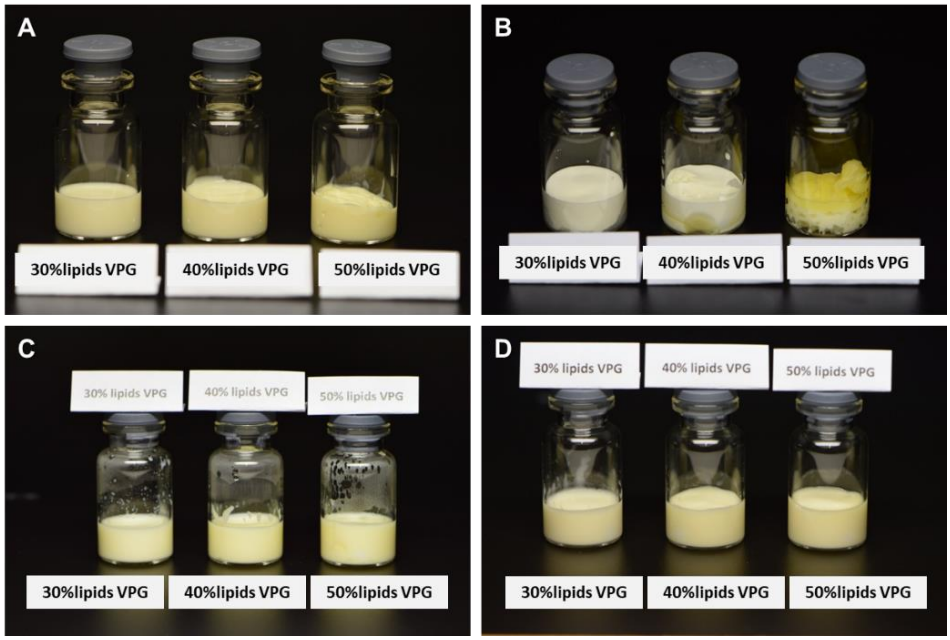

Figure 5-2 The freeze-drying and reconstitution of VPGs formulations. (A) fresh VPGs, (B) Lyophilized VPGs, (C) VPGs after reconstitution by magnetic stirring, (D) 15 days after reconstitution.

From representative samples of freeze-drying results (Figure 5-2), it can be concluded that the cake appearance was best for the 30% lipid VPG formulation. The 40% lipid VPG formulations were solid only in the upper half, and the appearance of 50% lipid VPG was disordered after freeze-drying. During the reconstitution step, the highly purified water was injected into the lyophilized glass vials (the lost weight during freeze-drying was substituted), and magnetic stirring for 45 min (from 100 rpm to 300 rpm, rotation rate was risen slowly) was used for the reconstitution (Figure 5-2, C). The samples were stored at 4 °C for 15 days, and the appearances of reconstituted VPGs of different lipid content groups were fine (Figure 5-2, D).

The applied lyophilization cycle is as described in Figure 5-3, and the product temperature and LyoRx (for freezing point determination and process control) were taken from the 40% lipid VPG samples. The LyoRx sensor could measure the electrical resistance of the sample, which can identify the thawing situation during the freeze-drying process [6,7]. In the freeze-drying process, the 40% lipid VPG could not be frozen completely, which is one of the reasons for the cake separation.

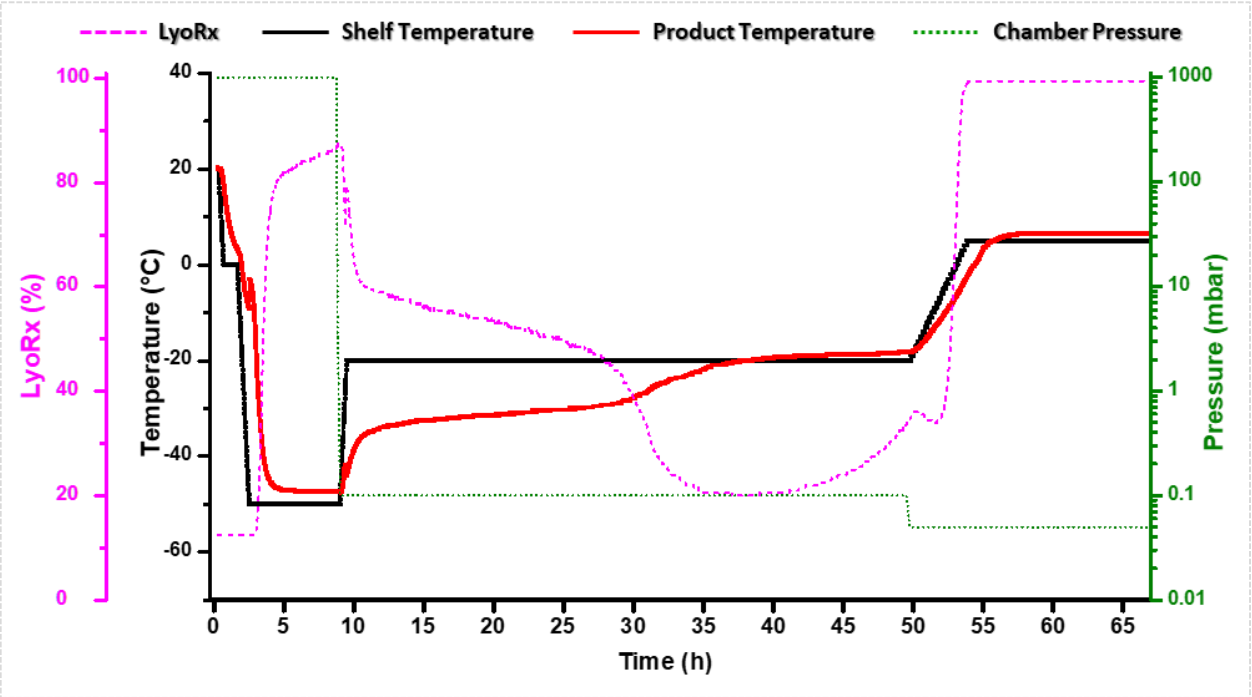

Figure 5-3 Time-dependent Pressure-Temperature-LyoRx profiles of freeze-drying of 40% lipid VPG by normal liquid freeze-drying method.

5.2.1.2 Freeze-drying for mAb loaded VPG (mAb@VPG) formulations

Because the high lipid content VPG formulations were not easy to lyophilize, the 30% lipid VPG was selected as a mAb delivery vehicle in the freeze-drying and reconstitution study, and following the results of Chapter 3, 5% (w/w) mAb was used as a feasible protein concentration.

Although the 30% VPG was used in freeze-drying because of it is good performance in previous experiments (compared to 40% and 50% lipid content VPG), the mAb loaded VPG good showed cake separation (Figure 5-4, A). Different methods were used to solve this problem, which includes addition of 5% (w/v) sucrose (Figure 5-4, B), or use of a large surface during freezing and subsequent sublimation (Figure 5-4, C), and finally the fast freezing with liquid nitrogen (Figure 5-4, D). The fast freezing with liquid nitrogen method was demonstrated to perform best to get feasible lyophilization cakes for the 5%mAb loaded 30%VPG formulation (5%mAb@30%VPG).

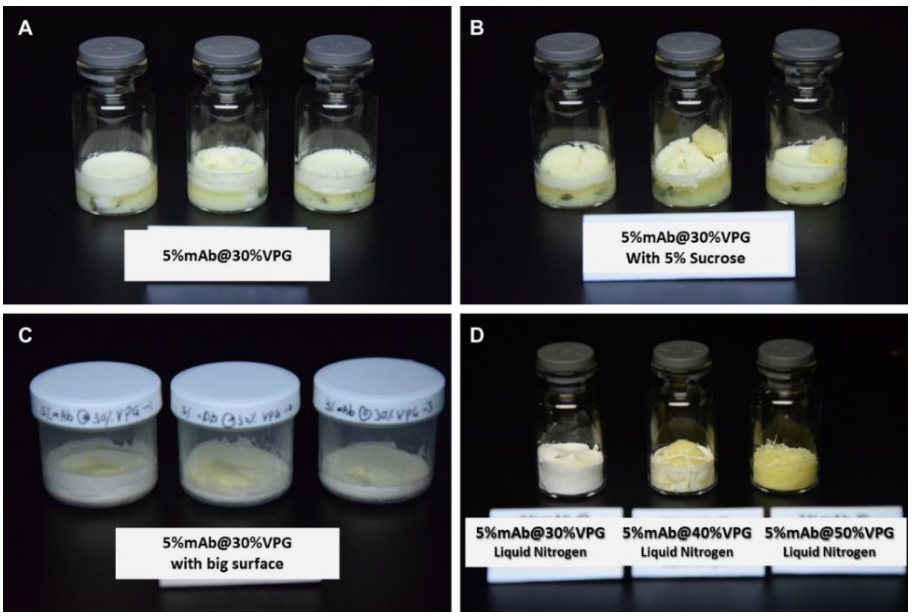

Figure 5-4 Different freeze-drying methods for mAb loaded VPG. (A) the cake in normal freeze-drying (B) the cake after normal freeze drying and addition of 5% sucrose (C) the freeze-drying in the large surface container, (D) the fast freezing with liquid nitrogen.

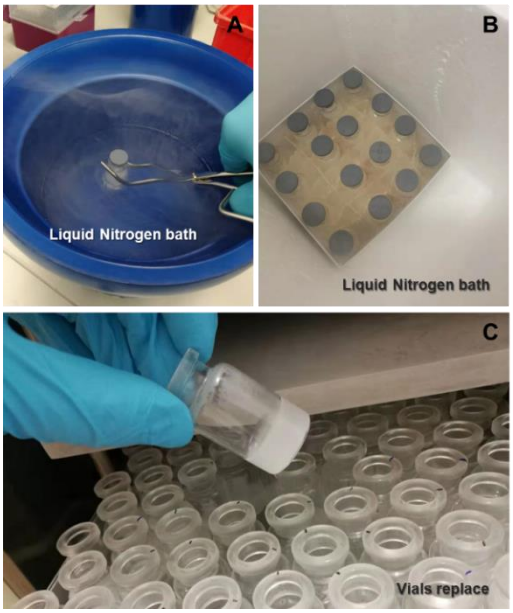

Figure 5-5. The fast freezing with liquid nitrogen before freeze-drying. (A) The single vial treated by fast freezing in liquid nitrogen. (B) The batch treatment treated by fast freezing in liquid nitrogen. (C) The frozen buffer vials were replaced by frozen 5%mAb@30%VPG vials in the freeze dryer.

The general operation steps of fast freezing with liquid nitrogen are shown in Figure 5-5. The 5%mAb@30%VPG formulation was treated with liquid nitrogen (Figure 5-5, A Single vials, B Batch processing.), and then replaced the frozen buffer vials in the freeze dryer in “VPG loading window“ (Figure 5-5, C) before the main freeze drying program started (The chamber pressure was decreased, and then the main program started).

After the fast-frozen mAb@VPG vials were loaded into the freeze-dryer, the chamber pressure was decreased to 0.1 mbar, and the shelf temperature was increased to -20 °C for 30 hours for primary drying. Then, the temperature increased to 4 °C for 12 hours of secondary drying. The time-dependent Pressure-Temperature-LyoRx profiles are given in Figure 5-6.

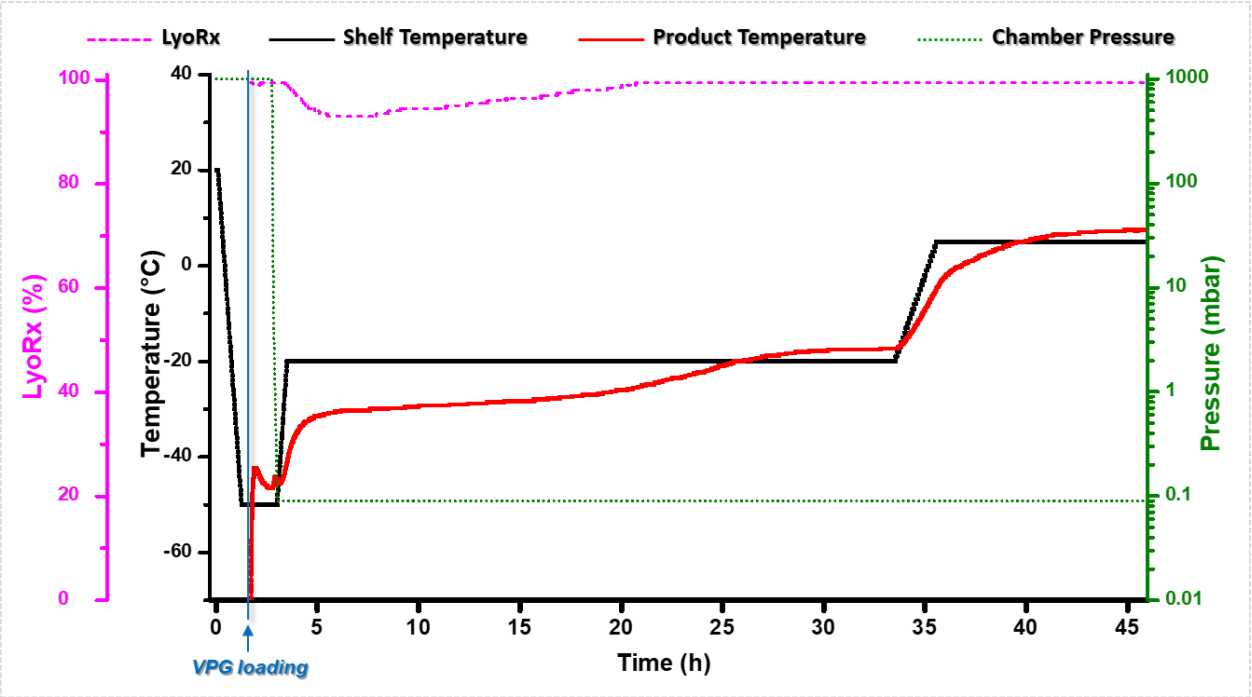

Figure 5-6 Time-dependent Pressure-Temperature-LyoRx profiles of VPG freeze-drying with liquid nitrogen treatment.

According to the process graphs of freeze-drying, the gel kept frozen during the whole freeze-drying progress (LyoRx), and the product temperature was stable during the freeze-drying. A feasible cake was obtained from fast-freezing with liquid nitrogen method.

Different lipid content VPGs with different mAb contents were tested using the fast freezing method (Figure 5-7). For the 5%mAb formulation, the highest lipid content for freeze-drying to obtain an intact cake is 32% (Figure 5-7, A). In the 3% and 1% mAb content formulations, the highest achievable lipid concentration was 34% (Figure 5-7, B, C). In the protein free VPG groups, the highest lipid concentration was 36% (Figure 5-7, D).

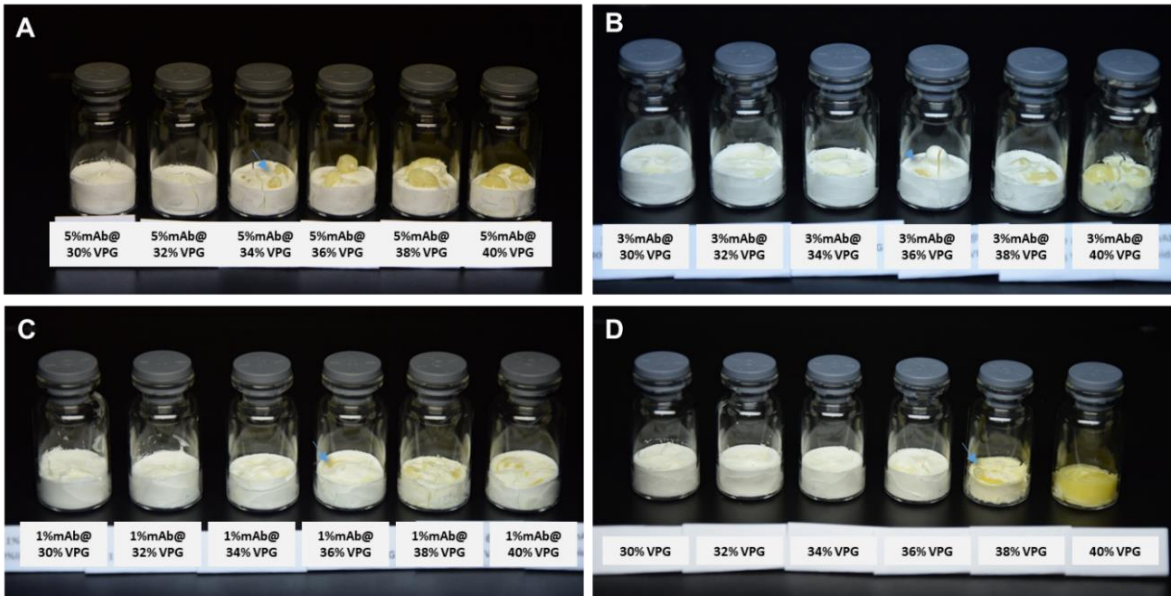

Figure 5-7 Lipid concentration screen with the fast freezing method in freeze-drying. (A) 5% mAb loaded VPGs with different lipid content, (B) 3% mAb loaded VPGs with different lipid content, (C) 1% mAb loaded VPGs with different lipid content, (D) VPGs with different lipid content.

1% and 5% (w/w) PVP was added, to raise the limit of achievable lipid concentration in lyophilized VPG formulations (Figure 5-8). After freeze-drying with the fast-freezing treatment, the cake of 5%mAb@VPG did not improve significantly.

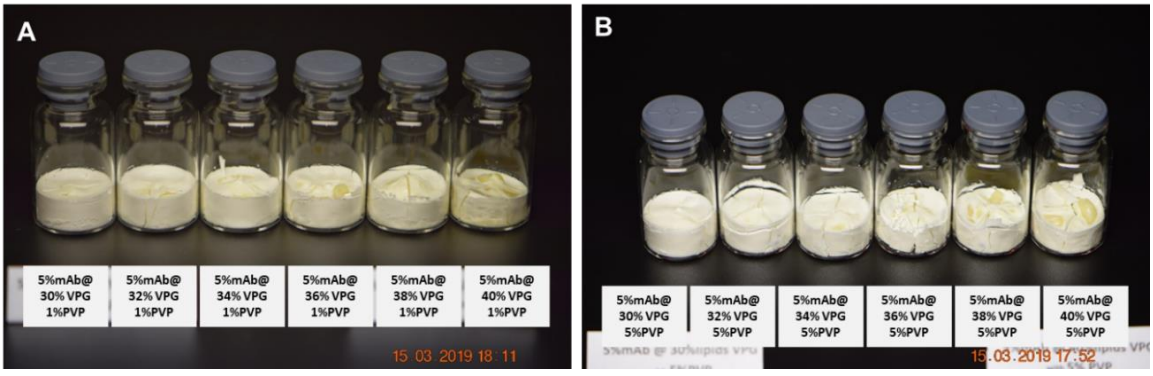

Figure 5-8 PVP was added to increase lipid concentration in freeze-drying. (A) 5%mAb@VPG with addition of 1% PVP, (B) 5%mAb@VPG with addition of 5% PVP.

A droplet freezing method was also tested: the different lipid content VPGs were added drop by drop into a glass vials filled with liquid nitrogen before freeze-drying (Figure 5-9) [8], and then the vials with frozen droplet VPGs were placed into freeze-dryer to replace the frozen vials with buffer as with the previous method in Figure 5-5. According to the results, this was a way to increase the lipid limit, but it is difficult to evaluate cake status, and more research should be performed for the evaluation of this method.

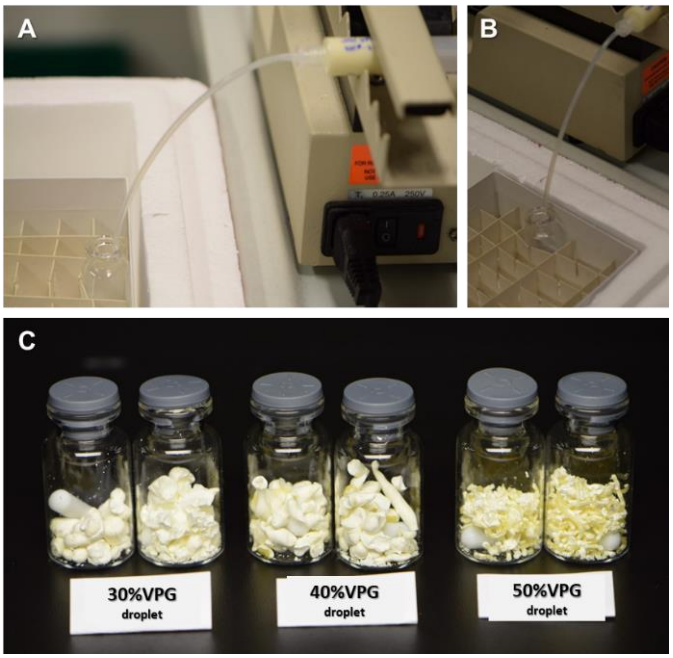

Figure 5-9 The droplet freeze-drying for VPG formulations. (A)(B) The droplet freeze-drying for VPGs from the syringe to the glass vials. (C) The appearance of VPGs from droplet after freeze-drying.

Residual moisture of 30%, 40%, and 50% lipid VPGs was measured by Headspace Karl-Fischer-Titration (Table 5-1).

	VPG	moisture content	
	(mg)	(µg)	%
30%VPG-1	3.8	6.4	0.168
30%VPG-2	5.9	8.3	0.141
40%VPG-1	7.5	39.6	0.528
40%VPG-2	15.8	118.1	0.747
50%VPG-1	18.8	169.3	0.901
50%VPG-2	16.7	155.7	0.932

Table 5-1 The moisture of VPGs after freeze-drying

Although the high lipid content VPG showed slightly higher moisture, the absolute water content of all lyophilized VPGs was low (Table 5-1), which means the reason for cake layer separation was not because of high residual water content.

The cake microstructures of lyophilized 5%mAb loaded VPG formulations was measured by SEM (Figure 5-10). The microstructures of different lipid content VPGs were significantly different. The cake surface of 30% VPG exhibited of lot of small pores (Figure 5-10 A), the surface of lyophilized 40% VPG was rather smooth (Figure 5-10 B), and lyophilized 50% VPG showed a very solid surface with several spherical lipid accumulations. (Figure 5-10 C).

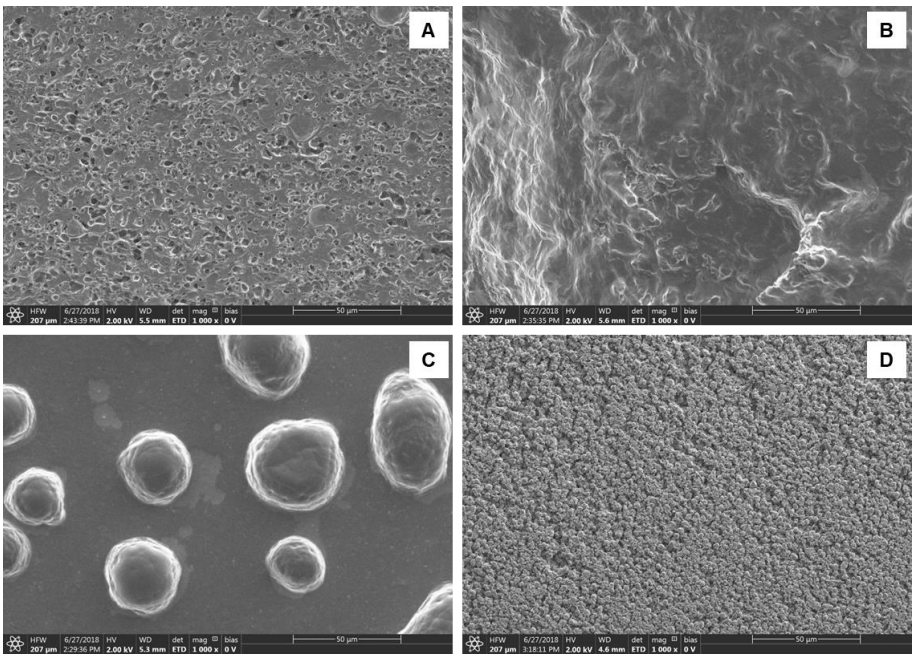

Figure 5-10 SEM micrographs of the surface after freeze-drying, (A) 5%mAb@30%VPG, (B) 5%mAb@40%VPG, (C) 5%mAb@50%VPG, (D) 20mM PBS (buffer solution of background).

5.2.2 The optical quality for gel stability of mAb loaded VPG formulations after freeze-drying

Batches of 5%mAb@30%VPG were prepared with the fast freezing (liquid nitrogen) method, and the respective water losses shown in table 5-2. A value of 1.827 g was calculated as the mean amount of injection water loss and this amount of what for injection was used for following reconstitutions.

	Gel sample (g)	whole glass vial with mAb@VPG		weight loss – water (g)
		before freeze-drying (g)	after freeze-drying (g)	
1	3.023	16.852	15.037	1.815
2	2.993	16.729	14.960	1.769
3	3.005	16.715	14.883	1.832
4	3.007	16.722	14.908	1.814
5	3.012	16.762	14.945	1.817
6	3.008	16.874	14.984	1.891
7	3.016	16.916	15.093	1.824
8	3.006	16.872	15.031	1.840
9	3.012	16.767	14.933	1.834
10	3.004	16.844	15.005	1.839
Mean	3.009	16.805	14.978	1.827

Table 5-2 the water loss of 5%mab@30%VPG during the freeze-drying

The batch preparation of 5%mAb@30%VPG was done for the long-term storage study (Figure 5-11), and the lyophilized 5%mAb@30%VPG formulations were stored at 4°C and 25°C, and samples were analyzed immediately after freeze-drying (week 0) and after 4, and 12 weeks of storage. The control references were the Wet 5%mAb@30%VPG, which were stored at the same conditions together with lyophilized samples. The correct position of the rubber stopper on the glass vials was with an Aluminum cap during the storage.

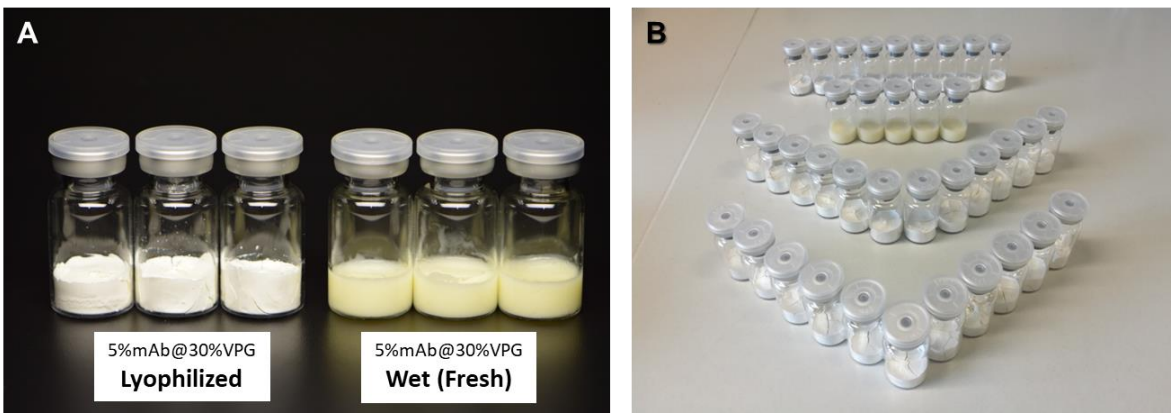

Figure 5-11 The batch preparation for the long-term stability of lyophilized 5%mAb@30%VPG

The lyophilized samples were reconstituted by magnetic stirring method before analysis. Figure 5-12 shows the appearance of Wet 5%mAb@30%VPG and Lyophilized-reconstitution (Lyo-Rec) 5%mAb@30%VPG after reconstitution at relevant test time points.

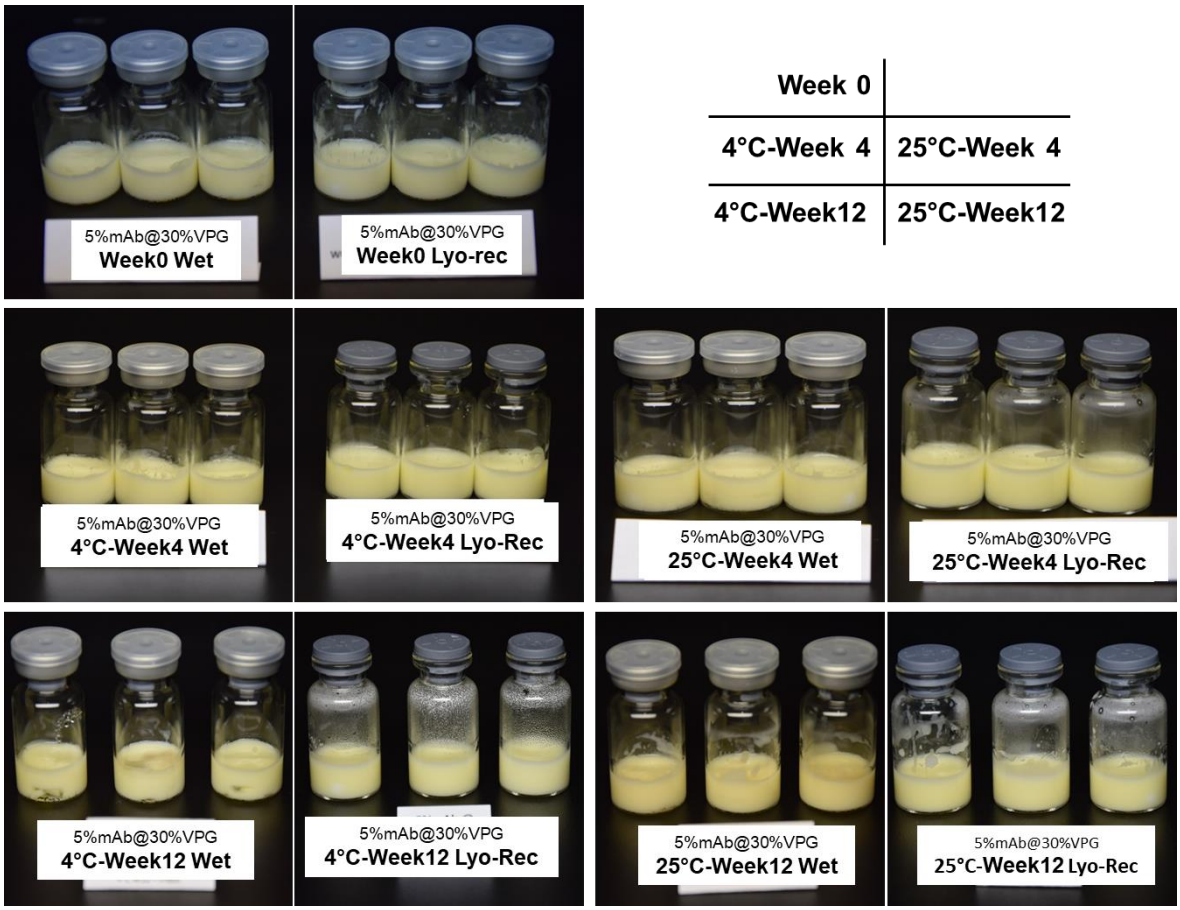

Figure 5-12 The appearance of lyophilized-reconstituted 5%mAb@30%VPGs in long-term storage study.

The Lyo-Rec 5%mAb@30%VPG appeared homogenous at all-time points. In comparison, lipid-phase separation occurred in the Wet control formulations at 4°C, after 12 weeks-12 of storage. Therefore, the long-term physical stability of lyophilized mAb loaded VPG can be considered superior, when compared to the Wet protein loaded VPG.

The microstructures of the 5%mAb@30%VPG surface after a 12 weeks of storage both at 4°C and 25°C are shown in figure 5-13. Compared to the fresh 5%mAb@30%VPG, small pores are distributed in all cakes, and the lyophilized 5%mAb@30%VPGs were stable enough after long-term storage both at 4°C and 25°C.

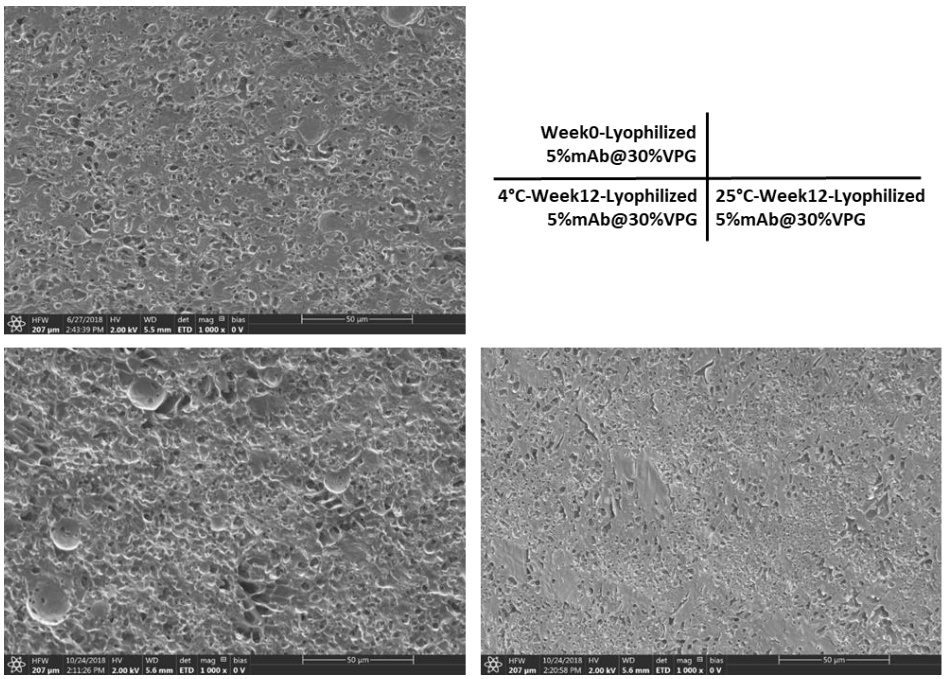

Figure 5-13 The micro-appearance of fresh and lyophilized 5%mAb@30VPG after 12 weeks by SEM.

According to the macro- and micro-appearance measurements, the morphology of lyophilized and reconstituted mAb loaded VPG formulations was stable during the long-term storage.

The viscosity and gel strength was measured by rotational Rheometer and the Texture Analyzer (TA) in Figure5-14, A, B (the detailed measurement methods are described in Chapter 2). The initial value of the 30% lipid VPGs was low (the viscosity of low lipid content groups is low, and more close to the property of liquid), and did not increase significantly in 4-weeks of storage. The viscosity and gel strength of wet VPG formulations were slightly increased after 12 weeks of storage, whereas the lyophilized groups showed stable viscosity values. TA was used to measure the gel strength (Figure 5-14), the basic values of 30% lipid VPG were low, and there was no significantly change after storage. According to the physical properties, the VPG was stable enough during the freeze-drying and subsequent reconstitution.

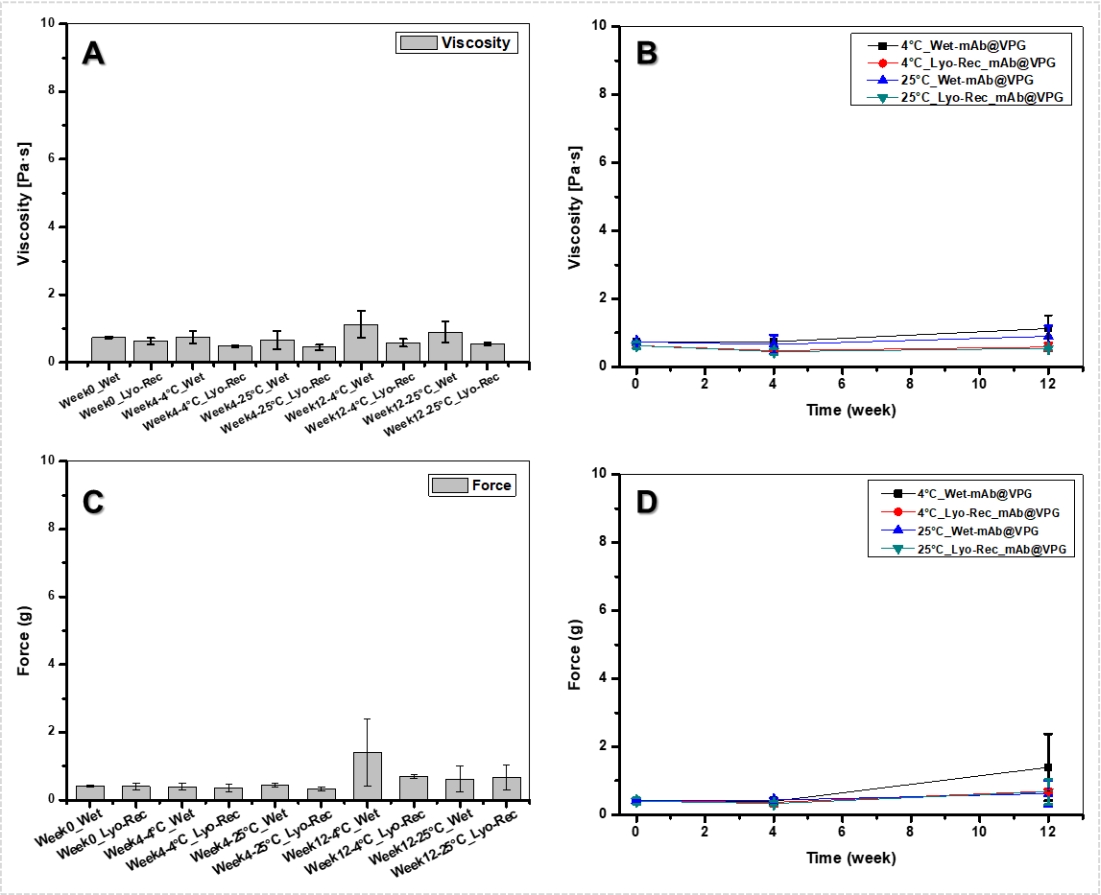

Figure 5-14. Viscosity and Texture Analyzer (TA) analysis of lyophilized-reconstituted 5%mAb@30%VPG after reconstitution in the long-term stability study.

5.2.3 Protein stability in the mAb loaded VPGs after freeze-drying and reconstitution

Protein stability is a very important part of biopharmaceutical research and development. In these experiments, several methods were used to investigate the protein stability of lyophilized and reconstituted 5%mAb@30%VPGs; the long-term stability was also investigated. The 5%mAb@30%VPG formulations were extracted with the method described in chapter 3, and the obtained aqueous protein solutions were analyzed by different methods.

5.2.3.1 Conformational stability by Near-UV Circular Dichroism (CD)

Near-UV of Circular Dichroism (CD) was used to investigate the tertiary structure (Figure 5-15). The mAb stock, extracted mAb stock, extracted 5%mAb@30%VPG, and the lyophilized-reconstituted and extracted 5%mAb@30%VPG were measured by Near-UV CD spectroscopy (250-320nm). The spectra showed the typical peaks of the native mAb, which include a positive peak around 295 nm and several negative peaks

between 280 and 250 nm, which means the freeze-drying and subsequent reconstitution did not strongly affect the tertiary structure of the mAb.

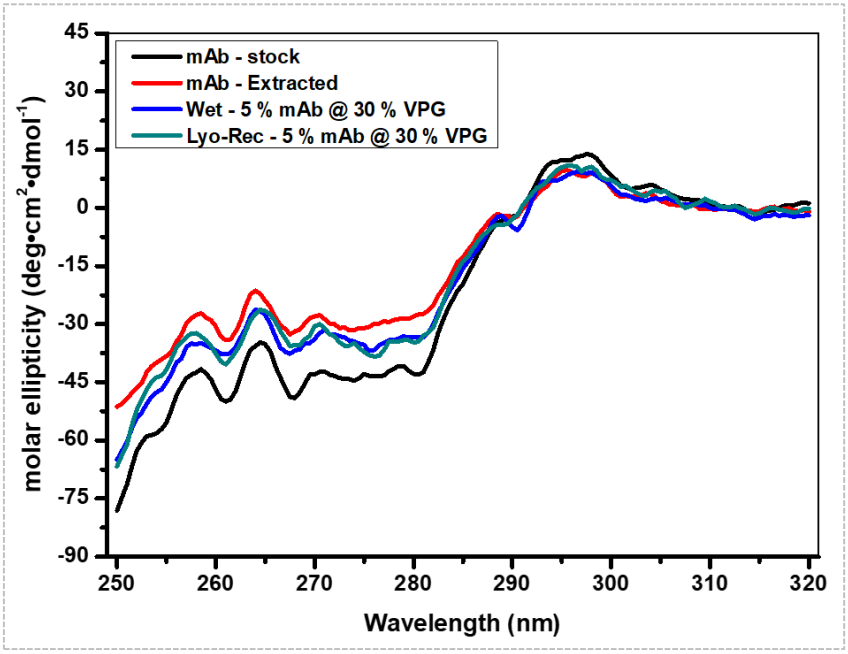

Figure 5-15 Near-UV Circular Dichroism of the extracted mAb.

5.2.3.2 Protein structure stability study for freeze-drying and reconstitution of mAb loaded VPG

SDS-PAGE is a powerful assay to detect protein aggregation. The aqueous protein solutions obtained after extraction, were analyzed by SDS-PAGE in no-reducing denaturing conditions (Figure 5-16). All electrophoresis bands of the samples matched the bands of the reference, the stock mAb solution. The SDS-PAGE did not indicate any aggregation that may have occurred during the freeze-drying, reconstitution, and long-term storage (both at 4°C and 25°C). In the week-12, the degradation was happening in wet storage groups, which both in 4°C and 25°C conditions one of three samples degraded.

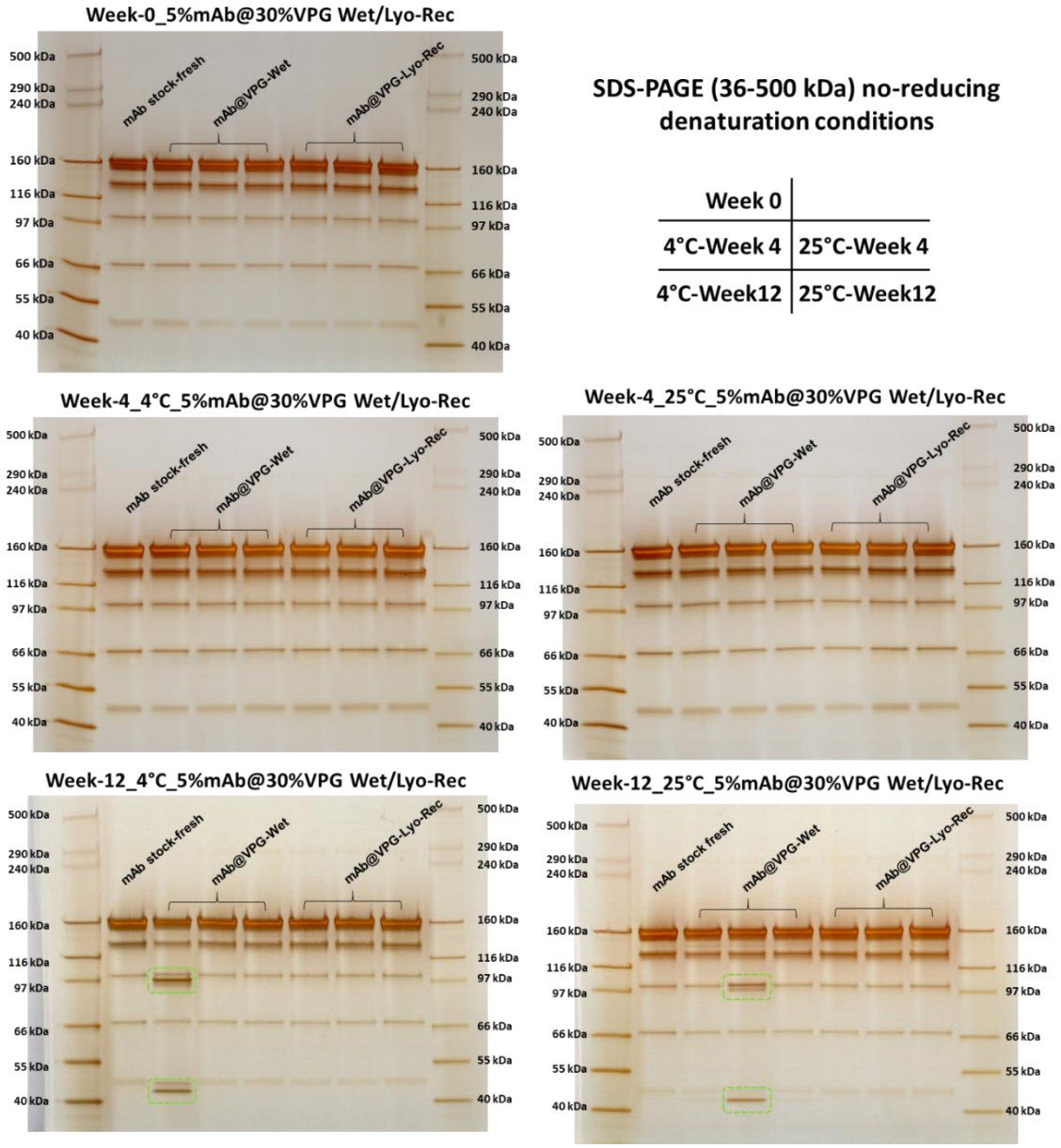

Figure 5-16 SDS-PAGE analysis for the Lyophilized-Reconstituted 5%mAb@30%VPG after reconstitution in the long-term storage stability study. (green frame: the fraction from protein degradation after storage).

5.2.3.3 Chromatographic analysis of protein stability by Size Exclusion High-Performance Liquid Chromatography (SE-HPLC)

The protein monomer content is an important parameter for protein formulations, and it was measured by SE-HPLC for quantitative analysis. The protein content in the extracted samples was determined by comparison to the standard curve. The detailed methods are described in Chapter 2. The chromatograms of reconstituted 5%mAb@30VPGs after long-term storage are shown as follows (Figure 5-17).

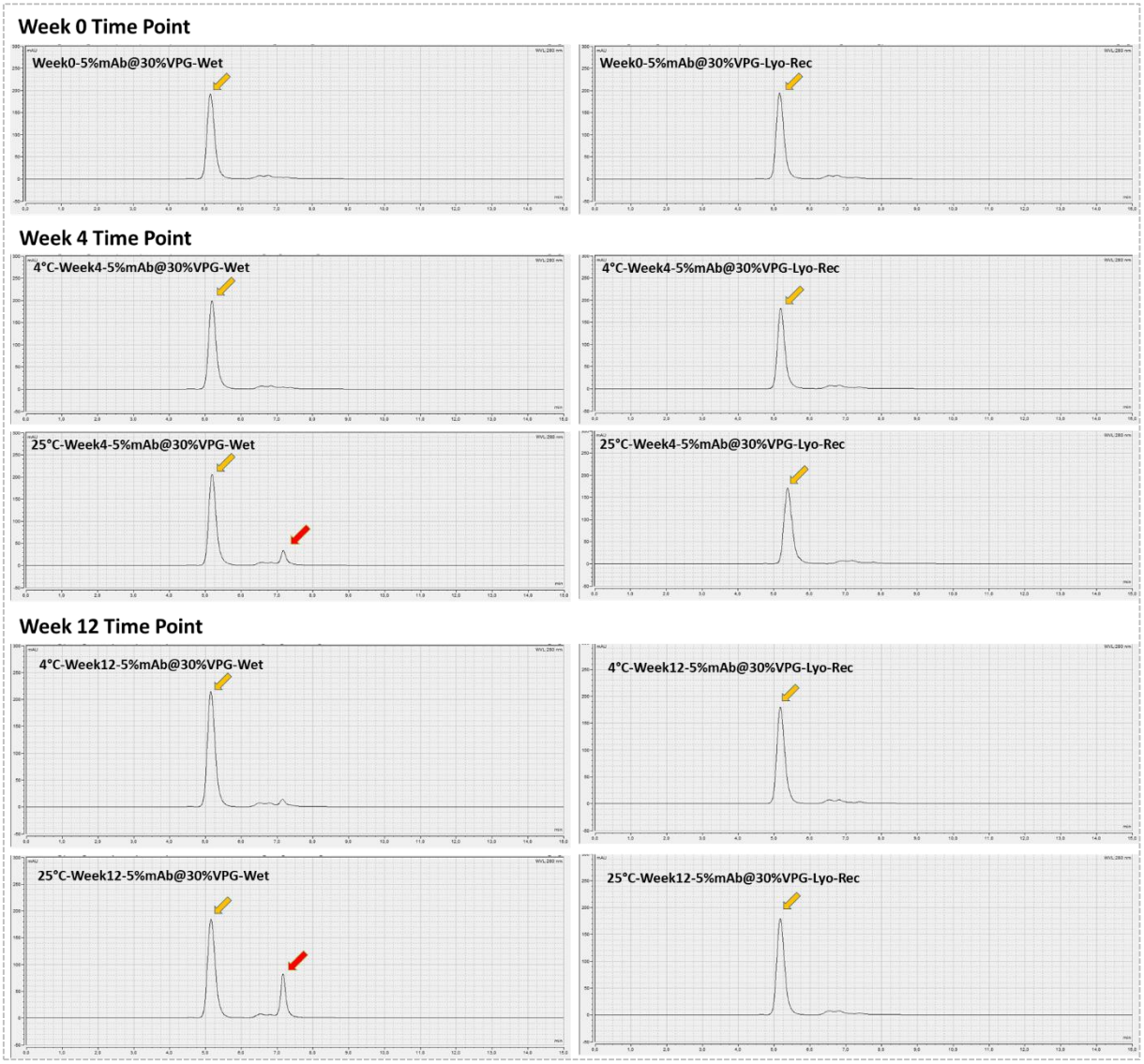

Figure 5-17 Chromatograms of SE-HPLC for 5%mAb@30%VPGs after long-term storage. Yellow arrow: monomer protein peak; Red arrow: degradation peak.

According to the results in Figure 5-17, some protein degradation happened in the 25°C-Wet-5%mAb@30%VPG formulation starting at week 4. At the same time, lyophilized formulations remained stable. The protein monomer content was determined by comparison to the standard curve (R^2=0.999), the protein monomer recovery concentrations were compared to the theoretical concentration of mAb within the VPG, which was used for the VPG preparation (Figure 5-18, A). The monomer protein content in wet VPGs changed significantly, which indicates insufficient stability of “liquid” VPGs during long-term storage. At the same time, the relative ratio of aggregates and monomer did not change much (Figure 5-18, B), which means the formation of soluble aggregates was not a primary reason for the instability, but degradation was the main reason for the loss of monomeric protein.

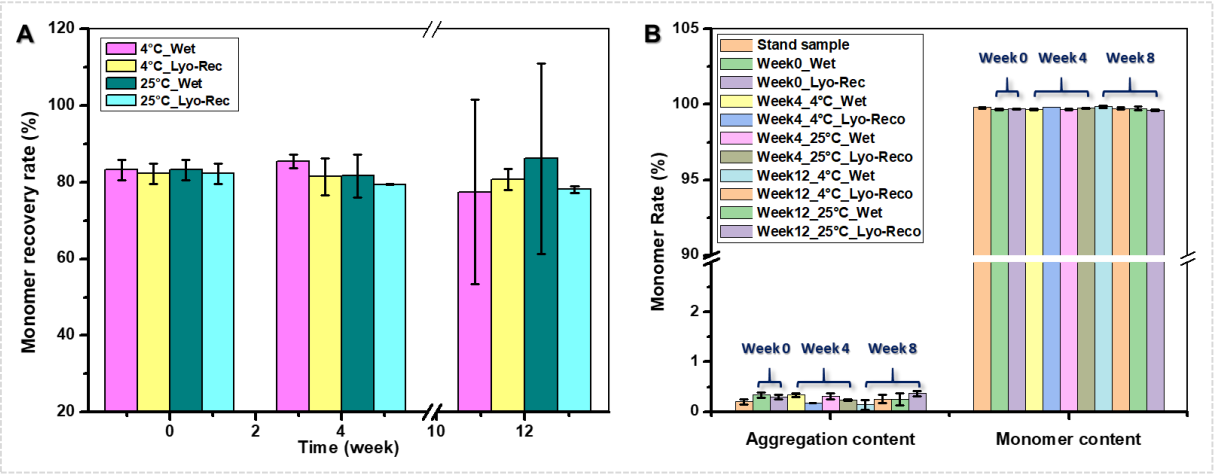

Figure 5-18 SE-HPLC measurements for 5%mAb@30%VPG during long-term storage after freeze-drying. (A) The recovery rate of monomeric antibody compared to the theoretical concentration after long-term storage. (B) The relative ratio of protein aggregates and monomer.

5.2.3.4 Chemical stability of the mAb measured by IEX-HPLC

The detection of charge variants of the mAb in long-term storage is an indicator for chemical instability. The protein solution was obtained after extraction, and after dilution with, 20 mM PBS pH7.4 (1:9 ratio) injected on the column. The detailed methods were described in chapter 2, and injections were performed in triplicates. The chromatograms of lyophilized 5%mAb@30VPGs after long-term storage are shown in Figure 5-19. As shown in the respective chromatogram, the main peak of wet-5%mAb@30%VPG decreased significantly after 12 weeks, and the acidic species increased at the same time, which means the “liquid” VPG was not suitable for the antibody stabilization at long-term storage in 25 °C. In contrast, freeze-drying enhanced the chemical stability in antibody loaded VPG formulation.

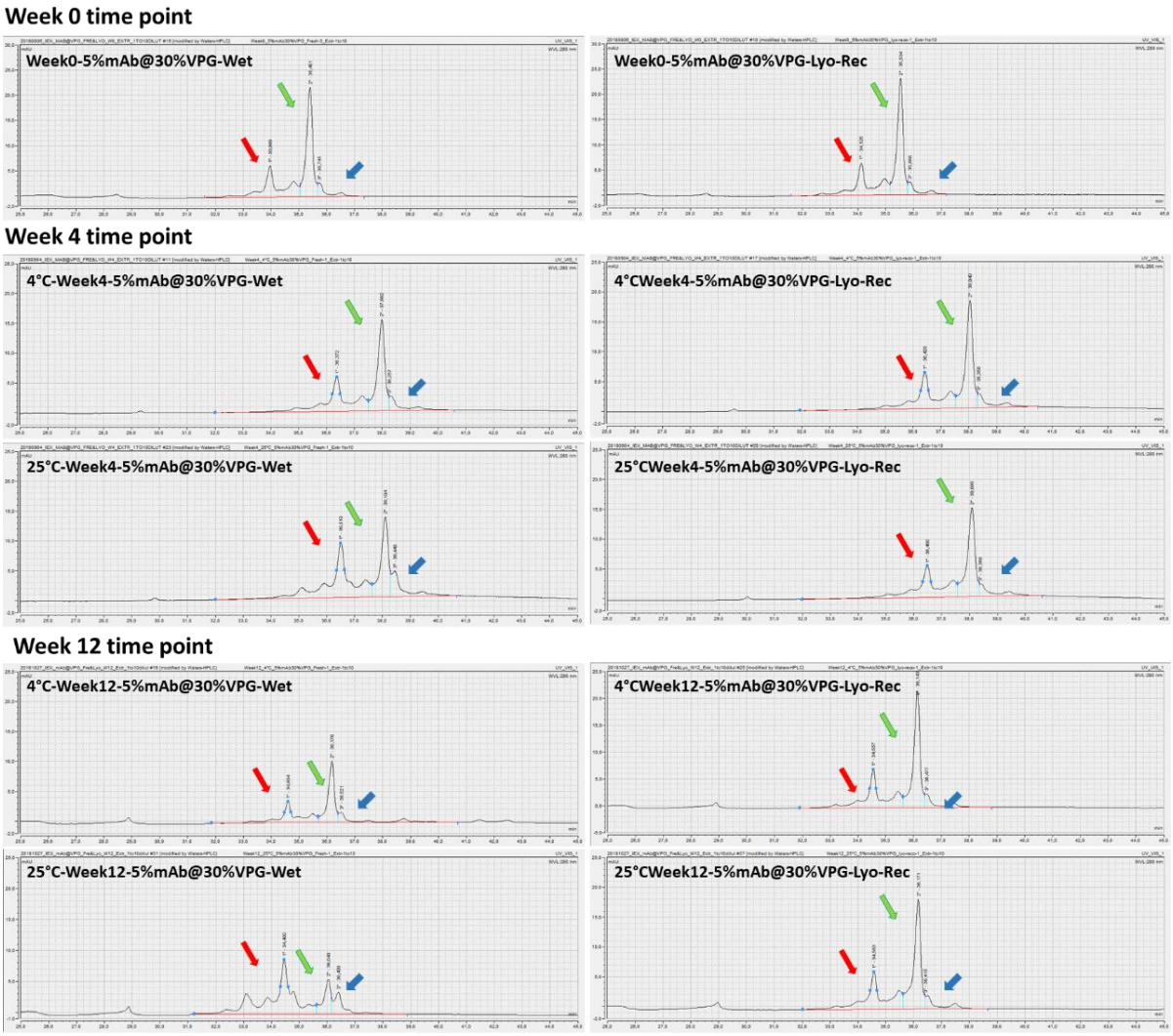

Figure 5-19 Chromatograms of IEX-HPLC for 5%mAb@30%VPG at long-term storage, Red arrow: Acidic species; Green arrow: Main Peak; Blue arrow: Basic species.

The peak area of the different charge variants was calculated, and the results are shown in Figure 5-20. According to the results of the charge variants the area of the main peak was slightly decreased after extraction, and freezing-drying and reconstitution did not affect chemical stability of the mAb. After 4 weeks of storage, the main peaks of the wet formulations stored at 25°C decreased and the acidic species increased. This trend is also visible after 12 weeks of storage (Figure 5-20 B, C). In contrast, Freeze-Drying enhanced the chemical stability of the mAb after storage, both at 4 °C and 25 °C.

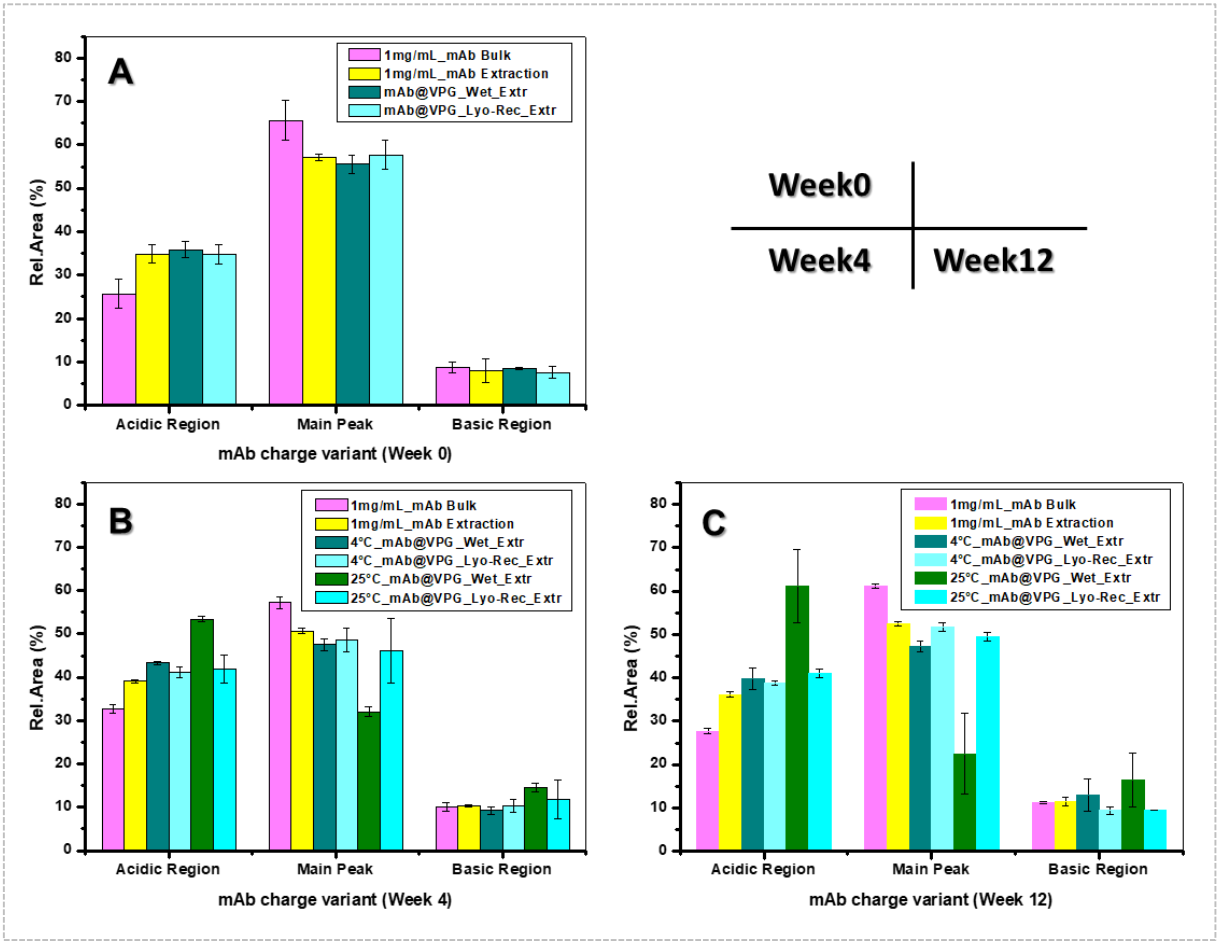

Figure 5-20 Percentage of charge variant after reconstitution of the lyophilized 5%mAb@30%VPG with highly purified water. (A) protein charge variants in week-0, (B) protein charge variants in week 4, (C) protein charge variants in week-12.

According to these results, freeze-drying is a feasible approach to improve the chemical stability of the mAb during the long-term storage.

5.2.3.5 Bio-affinity stability analysis of mAb loaded VPGs in long-term storage.

The Bio-Layer interferometry is an efficient method to measure the bio-affinity of the respective protein, and also protein concentration could be quantitatively analyzed. For the antibody, the Fc and Fab regions were two main active regions for bio-functions, and these two regions were measured by Protein A and FAB2G biosensors separately, and the fresh mAb stock was used to make the standard curves for measurement. The week-0 and week-12 time points were measured by Bio-Layer Interferometry (BLItz® system), the detailed method was described in chapter 2, and all samples were measured as triplicates (Figure 5-21). At constant absolute protein concentration, the apparent the Fc region active concentration changed both in wet and freeze-dried

formulations (Figure 5-21, A), which means the Fc region was sensitive to degradation in both wet and lyophilized formulations. At the same time, the Fab region remained stable in the freeze-dried VPGs (Figure 5-21, B), while the Fab region in wet mAb loaded VPGs was unstable. This illustrates that lyophilization could protect the Fab region of the antibody against degradation in long-term storage.

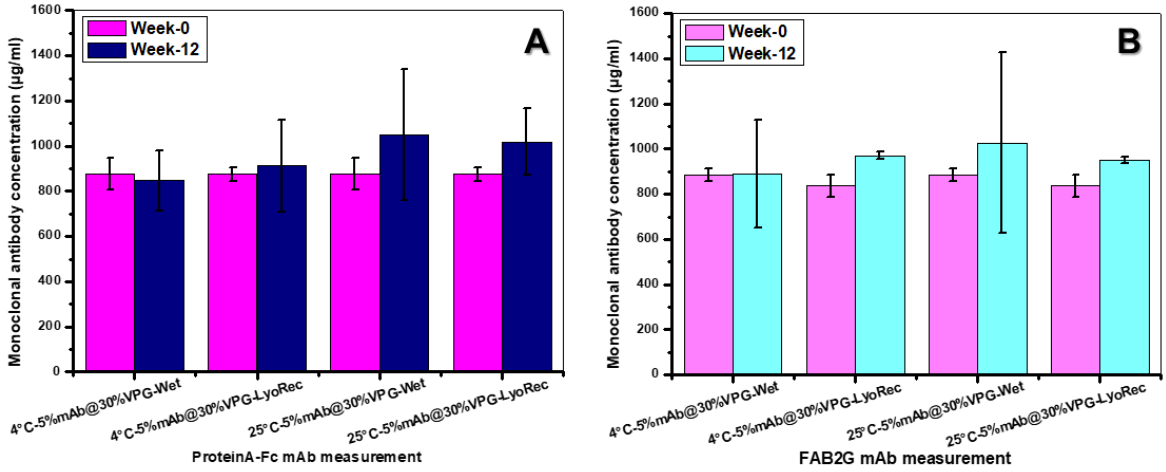

Figure 5-21 The Fc (A) and Fab (B) region binding curves of mAb of freeze-dried VPGs during long-term storage.

According to the bio-affinity analysis, lyophilization demonstrated good performance to protect protein bio-affinity capability in VPG formulations.

5.2.3.6 Lipid stability analysis by the lipid peroxidation (MDA) assay kit

Lipids are the main component of VPG formulations, and their chemical stability is also an important parameter during the long-term stability study. The lipid peroxidation was measured during the long-term storage by the lipid peroxidation (MDA) assay kit (MAK085, Sigma-Aldrich). The levels of malondialdehyde (product of lipid peroxidation) were measured during the long-term storage study (Figure 5-22).

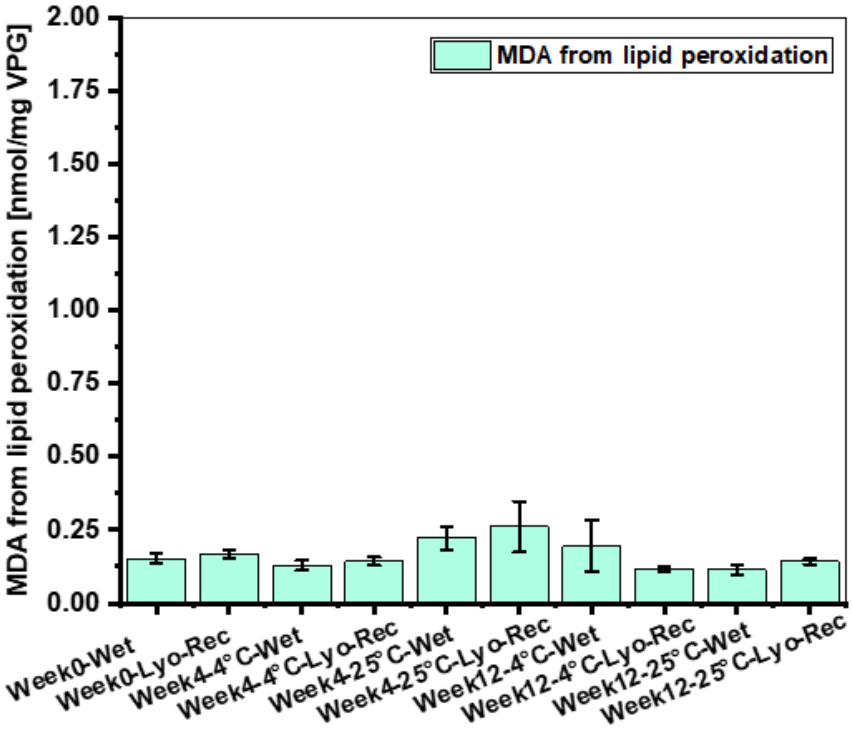

Figure 5-22 MDA measurement for lipid stability during the 5%mAb@30%VPG long-term storage study.

According to the MDA measurement, the MDA concentrations in the whole long-term storage study were kept at a very low level, which illustrates the peroxidation was not a stability-limiting factor in protein loaded VPG formulation.

5.2.4 The release performance of mAb loaded VPG formulations in long-term storage

The release behavior was an important parameter for VPG formulations. During the long-term storage, the release was tested in the Small Filter Holder (SFH) model for 21 days and the mAb and lipid concentration in the released fractions were measured by RP-HPLC and the phospholipid test assay kit. The detailed methods were described in chapter 2. The release behavior is shown in Figure 5-23.

According to the release measurements, the mAb and lipid release behavior were similar both in the wet and lyophilized formulations (Figure 5-23, A, B), which means the freeze-drying did not affect the release performance of mAb loaded VPG formulations.

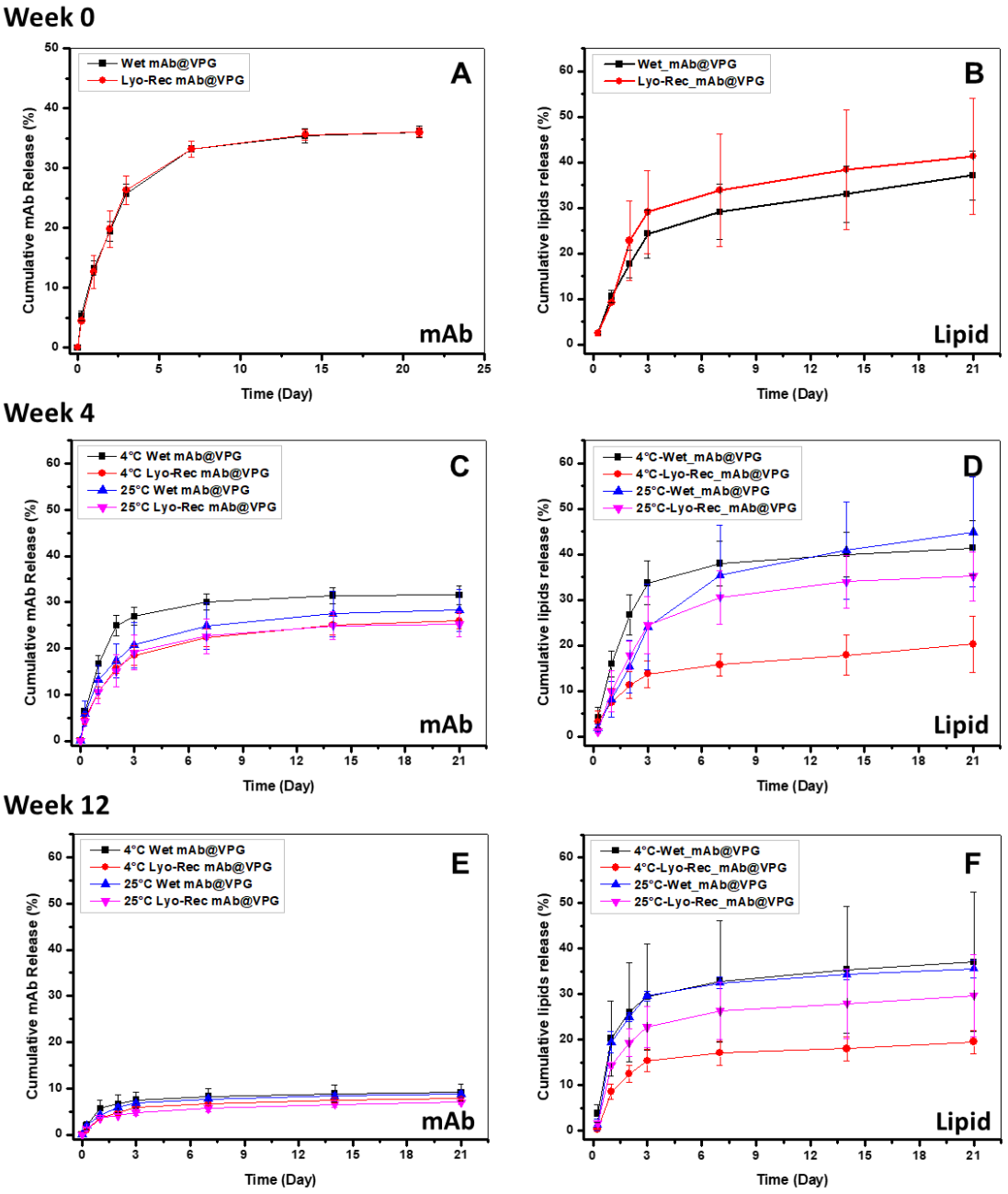

Figure 5-23. The mAb and lipid release performance with SFH model of 5%mAb@30%VPG in long-term storage study. (A) The mAb release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG in week-0. (B) The lipid release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG in week-0. (C) The mAb release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG after 4 weeks' storage. (D) The lipid release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG after 4 weeks' storage. (E) The mAb release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG after 12 weeks' storage. (F) The lipid release behaviors of Wet and Lyo-Rec 5%mAb@30%VPG after 12 weeks' storage.

The release rate of mAb and lipid decreased together with storage time, and the release rate of the lyophilized formulation was decreased more after 4 weeks of storage. The mAb release rate decreased for all formulations after 12 weeks of storage, but the lipid release rate was similar to the situation after 4 weeks of storage. At the same time, in the fresh mAb loaded VPG groups, the release rate of mAb and lipid decreased after

the cumulative release close to 40% after 2 weeks. Compared with the FITC-Dextran loaded VPG formulation, the viscosity of antibody loaded VPG was higher, which would bring higher gel stability to the mAb loaded VPG formulation.

5.2.5 Summary for storage study of freeze-dried mAb loaded VPG

The fast freezing freeze-drying method was developed for mAb loaded VPG formulations, and a cake with good appearance was achieved after method optimization. The magnetic stirring with a small stir bar in the dried vial was demonstrated to be an easy and effective method for the VPG reconstitution. At the same time, the protein stability was tested in long-term storage after lyophilization, which includes the structural, chemical, and bio-affinity stability. Compared with the wet stored mAb loaded VPGs, the freeze-dried formulations stabilized the protein in long-term storage. The lipid chemical stability was also measured, and no significant peroxidation occurred in VPGs. The release rate decreased over time in the freeze-dried formulations, and the possible reason was the interaction between the antibody and lipid in microstructure. With that, we must conclude that despite the fact of good protein stabilization, it is not yet possible to store VPGs in freeze-dried from the longer periods as the release properties are partly lost over time, and it will be very important topic for VPG formulation studies in future.

5.3 Stability study on frozen state storage of monoclonal antibody loaded VPGs

5.3.1 Freezing and thawing of the monoclonal antibody loaded VPG formulations

The frozen state is a widely used method for sample storage in research and development, and it was here applied to the monoclonal antibody (mAb) loaded VPGs. The 5%mAb@30%VPG, 5%mAb@35%VPG, 5%mAb@40%VPG, and 5%mAb@45%VPG, were tested in 4°C, -20°C, and -80°C storage conditions (Table 5-3). 3g of each sample were filled into the glass vials (n=3) with small magnetic stirring bar like the freeze-dried formulations, and then all samples were stored at 4°C, -20°C, and -80°C separately (n=3). After 4 weeks of storage at different conditions, the samples were analyzed. The samples stored at -20°C and -80°C were thawed at 4°C overnight, and then all sample were mixed by magnetic stirring for 45min before analysis.

	30% lipid VPG (w/w)	35% lipid VPG (w/w)	40% lipid VPG (w/w)	45% lipid VPG (w/w)
Wet (4°C)	**5%mAb@30%VPG**	**5%mAb@35%VPG**	**5%mAb@40%VPG**	**5%mAb@45%VPG**
Frozen (- 20°C)	**5%mAb@30%VPG**	**5%mAb@35%VPG**	**5%mAb@40%VPG**	**5%mAb@45%VPG**
Frozen (- 80°C)	**5%mAb@30%VPG**	**5%mAb@35%VPG**	**5%mAb@40%VPG**	**5%mAb@45%VPG**

Table 5-3. The formulations in the mAb loaded VPG in frozen storage study (n=3)

5.3.2 The appearance of mAb loaded VPG after frozen state storage

The mAb loaded VPG formulations were ready for measurement after thawing and mixing. The general appearance of formulations is shown in Figure 5-24. The appearance of all gels was similar, and the frozen state and re-mixing did not affect the appearance of the VPG formulations.

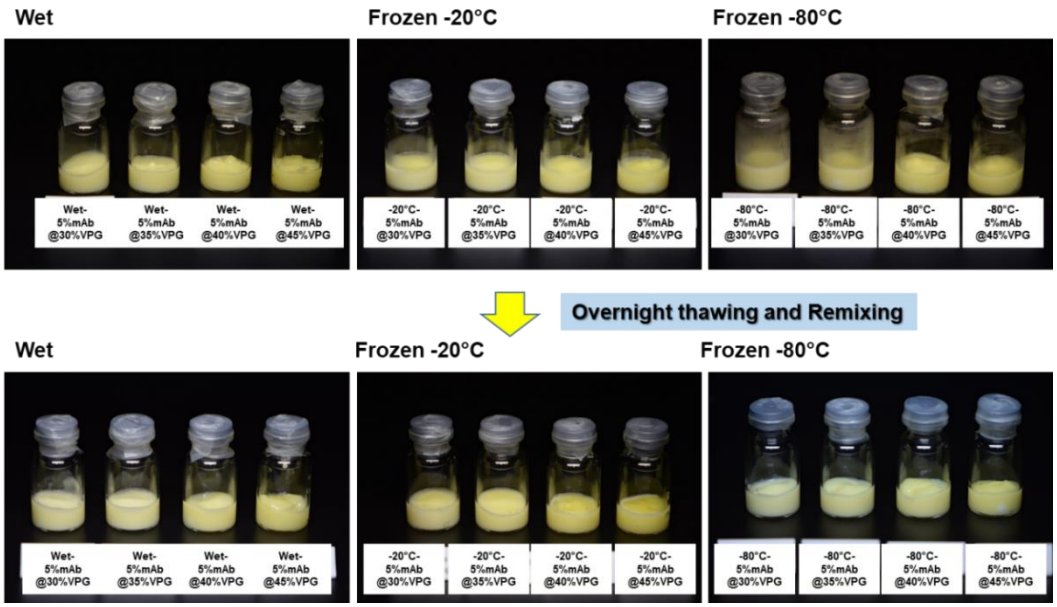

Figure 5-24 The appearance of mAb loaded VPGs in the frozen state storage study

The viscosity and gel strength were measured by the rotational Rheometer and Texture Analyzer (Figure 5-25). According to the results, the mechanical properties of low lipid formulations (30% and 35% VPG) were similar, and there was no difference between wet and frozen mAb loaded VPGs. In contrast, the viscosity and gel strength of high lipid content formulations (40% and 45% VPG) were decreased significantly after frozen storage. The possible reason could be a change in the microstructure, that happened by reorganisation of molecules during the crystallization process.

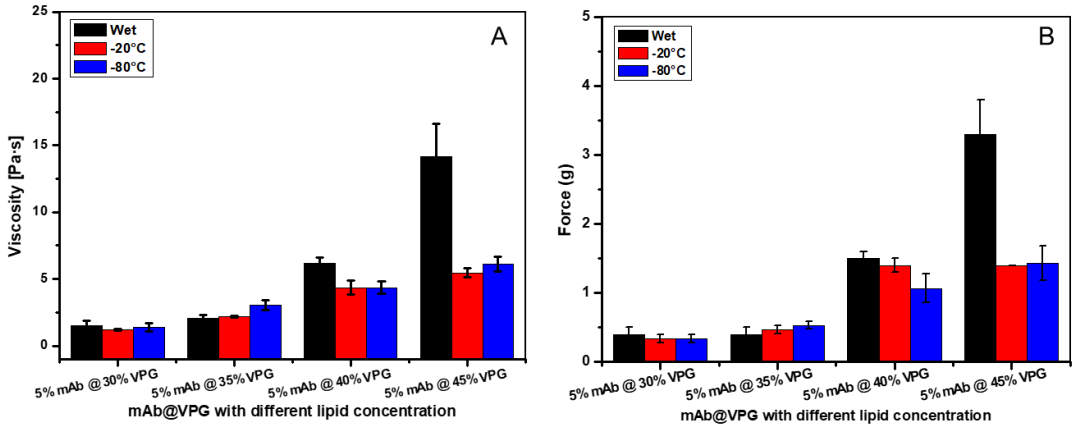

Figure 5-25 The Viscosity (A) and Texture Analyzer analysis (B) of frozen mAb loaded30%, 35%,40%, & 45%VPG in the frozen state stability study.

5.3.3 Protein stability of mAb loaded VPG in the frozen state storage study

The protein stability measurement was the core part of the stability study, and for the frozen mAb loaded VPG, the stability measurement includes the physical stability,

chemical stability, and bio-affinity stability. Before these measurements, the purified mAb aqueous solutions were obtained by the extraction method described in chapter 3.

5.3.3.1 Protein structural stability measurements

The protein was analyzed by SDS-PAGE, and the results are shown in Figure 5-26. According to the SDS-PAGE results, no visible aggregation occurred in both wet and frozen formulations.

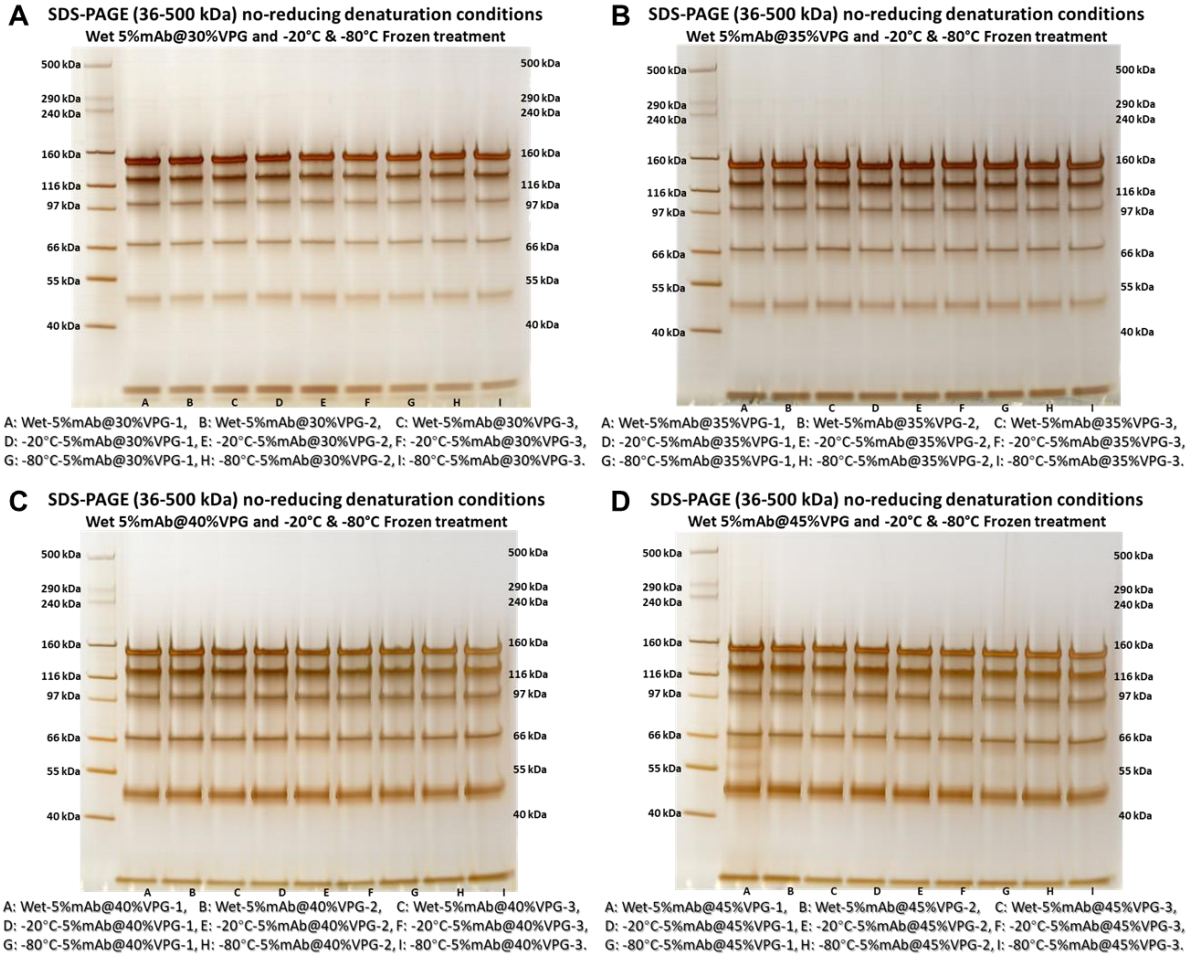

Figure 5-26 SDS-PAGE analysis for the frozen 5%mAb@30%, 35%, 40% & 45%VPG in the frozen state storage study. (A) mAb loaded 30%VPG in Wet, - 20°C, and - 80°C. (B) mAb loaded 35%VPG in Wet, - 20°C, and - 80°C. (C) mAb loaded 40%VPG in Wet, - 20°C, and - 80°C. (D) mAb loaded 45%VPG in Wet, - 20°C, and - 80°C.

The SE-HPLC is a quantitative analysis method for protein aggregation and degradation. The mAb sample solutions were analyzed with SE-HPLC for protein monomer content measurement, and the results are shown in Figure 5-27.

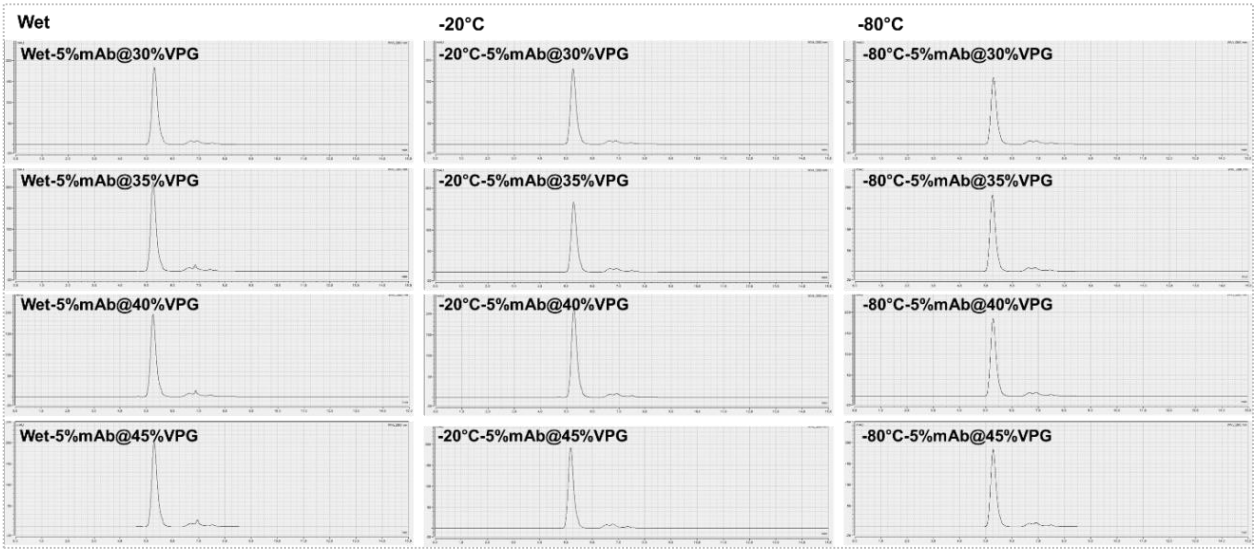

Figure 5-27 Chromatograms of SE-HPLC for mAb of 5%mAb@30%, 35%, 40%, & 45%VPG in the frozen state storage study.

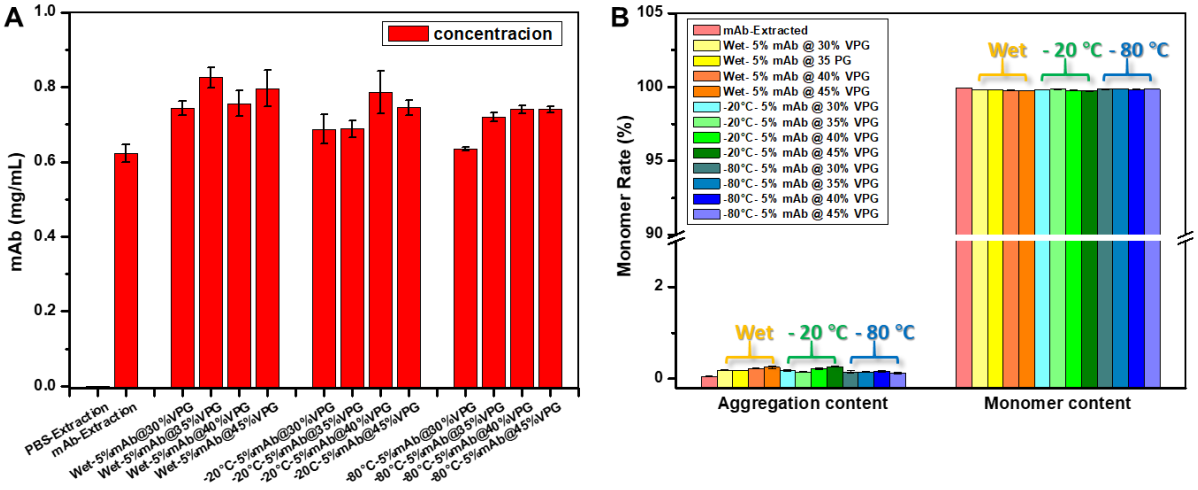

Figure 5-28 SE-HPLC measurement for 5%mAb@30%, 35%, 40% & 45% VPG during frozen storage. (A) The recovery rate of the monomer antibody compared to the theoretical concentration in frozen storage. (B) The relative concentration of protein aggregation and monomer.

After calculation with the standard curve (R^2=999), the protein monomer recovery concentrations were placed in Figure 5-28 A, and the monomer concentration in VPG did not change significantly. The relative ratio of aggregates and monomer did also not change (Figure 5-28, B). According to these results, the frozen storage (both in -20°C and -80°C) and subsequent thawing and mixing did not cause significant protein aggregation or degradation.

5.3.3.2 Protein chemical stability measurements

The mAb aqueous solutions were measured by IEX-HPLC for mAb charge variant analysis, and the detail methods were described in chapter 2. All samples were

calculated and displayed by comparison to the stock solution, and all samples were injected in triplicates.

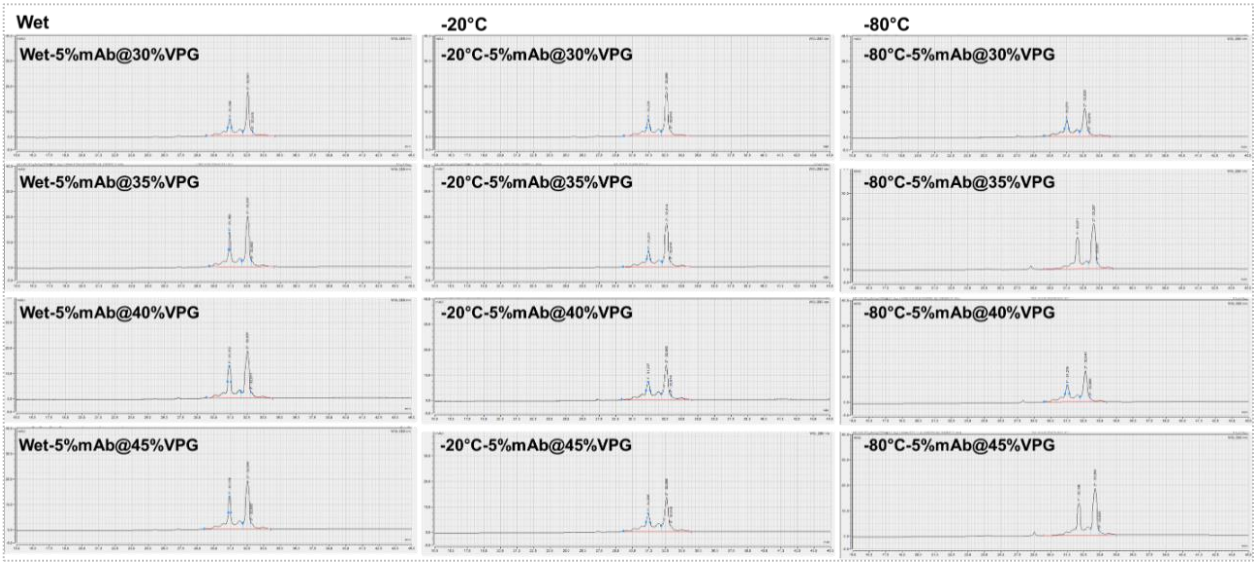

Figure 5-29 Chromatograms of IEX-HPLC for mAb of 5%mAb@30%, 35%, 40%, & 45%VPG in the frozen state storage.

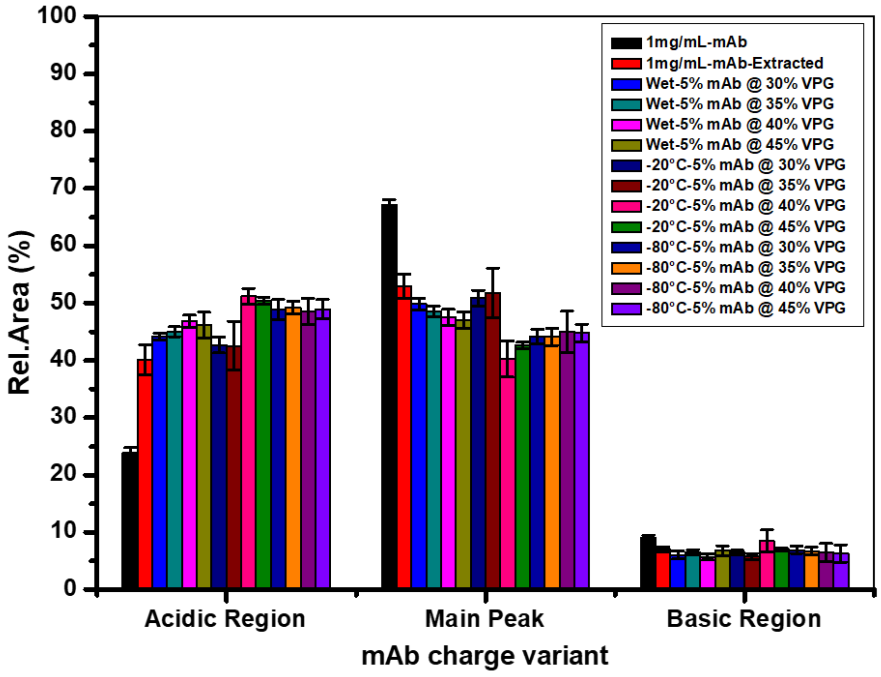

Figure 5-30 Percentage of charge variants after thawing and re-mixing of mAb loaded VPG in frozen storage.

Figure 5-30 illustrates that the main charge variant of mAb extracted was distributed from 49.8 to 40.2%, the general distribution of different groups was similar, and 30% and 35% VPGs in -20°C performed better compered to all other frozen samples.

5.3.3.3 The bio-affinity stability analysis of mAb loaded VPG after frozen storage.

The Qctet^{TM} system was used for the mAb bio-layer interferometry analysis in the frozen state storage analysis of mAb loaded VPG formulations. The Fc and Fab region of mAb were both measured by the BLI analysis. The detailed method was described in chapter 2, all sample were measured in triplicates (Figure 5-31). According to the BLI measurements, the Fc and Fab region active concentration was similar to the extracted mAb groups, and the frozen and thawing operation did not negatively affect bio-affinity capability of the mAb.

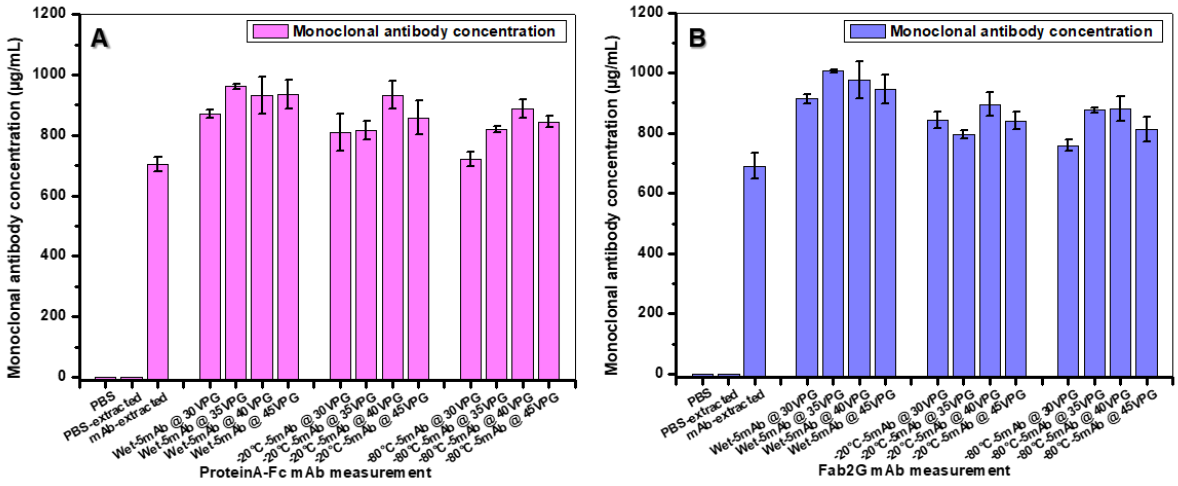

Figure 5-31 The Fc (A) and Fab (B) region binding curves of mAb of freeze-drying VPG in frozen storage.

According to the investigations (SDS-PAGE, SE-HPLC, IEX-HPLC, and BLI etc.), the structural, chemical, and bio-affinity stability of the mAb was feasible in the frozen state storage and subsequent thawing. The rate constant of the most chemical reactions was reduced in the frozen state.

5.3.4 Summary on stability studies of frozen state storage of mAb loaded VPG

The Frozen state storage method also demonstrated a feasible possibility to store the mAb loaded VPG formulations with good protein stability protection. The VPG physical properties, protein structure stability, protein chemical stability, and protein bio-affinity stability were measured, which showed good protection for the protein molecules. According to the operation side, it was supporting an easy way to store the protein loaded VPG formulations for the research and development.

5.4 Conclusion

The storage stability is an important part of formulation development. Summarizing the results of the long-term storage studies of Chapter 5, one can say that the long-term storage of mAb loaded VPG formulation was possible. Different methods have been tested, which included the storage of freeze dried products and frozen state storage.

First, the fast freezing freeze-drying method was developed for mAb loaded VPG formulations; the cake appearance of fast freeze-drying (liquid nitrogen) was better than for the normal freeze-drying method. The magnetic stirring was demonstrated to be an effective method for the reconstitution of the VPGs. Although the reconstitution times were nearly one hour totally, the reconstitution time is acceptable in the development phase. In the future, other methods could be tested for a faster reconstitution of lyophilized VPG formulations.

Second, protein stability in freeze dried VPGs was tested in long-term storage, which includes the structural, chemical, and bio-affinity stability. The mAb was not stable enough in “liquid” wet VPG during 12 weeks’ long-term storage, where the structure, chemical, and bio-affinity stabilities were all decreased. On the other hand, the freeze-dried formulations stabilized the protein over long-term storage, the mAb encapsulated by VPG showed good stability performance even at room temperature (25°C) after 12 weeks’ storage. At the same time, the lipid chemical stability was also measured, and no significant peroxidation occurred in VPGs. The release rate decreased in the freeze-dried formulations overtime, and the longer storage caused a lower release rate. This phenomenon is a serious drawback for this storage form, and the possible reason was the interaction between the protein and lipid in the microstructure.

Third, the frozen state storage method also demonstrated a feasible possibility to store the mAb loaded VPG formulations directly, with good protein stability protection. Compared with the freeze-drying, the froze storage could be an easy technical way to store the protein loaded VPG formulations. This method also has the release rate decrease problem need to solve before application, and some structure changing may happen in the frozen process.

5.5 Reference

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Chapter 6 The scale-up studies for VPGs

6.1 Introduction

VPG is a semi-solid depot formulation suited for both hydrophilic and hydrophobic drugs, and VPG also has special properties for long term release performance. Currently, most researches were focused on the good biocompatibility, sustained release behavior, and therapeutic performance of VPG formulations[1,2], and only small volume samples were investigated. The scale-up of manufacturing methods is an important step close to the application, and different manufacturing methods have been tested for large volume VPG preparation, and storage experiments have also been done for comparison between groups about different manufactured samples.

6.2 Scale-up of different manufacturing methods for VPGs

6.2.1 Method optimization for VPG scale-up

During the first research work about VPG by Martin Brandl et al.,[3] the initial VPG preparation method was High-Pressure Homogenization (HPH), and then the Dual Asymmetric Centrifuge (DAC) was brought into the VPG preparation by Ulrich Massing et al [4] for good quality formulations, and more currently the Magnetic Stirring (MS) and Extruder were also used for the VPG preparation[5,6]. The VPG prepared by DAC has shown good performance both for hydrophilic and hydrophobic drug encapsulation with good sustained release capability. The DAC preparation method was widely used for protein, peptide, and small molecular drugs. The VPG volume of single batch preparation by DAC was limited (≤ 4 g) during the laboratory operation, now different methods were tested for VPG scale-up manufacture (≥ 20 g). During these works, the HPH, DAC, MS, and Extruder were used for large volume preparation, and then the VPG stability performance from the different sources was compared. The basic methods have been described in Chapter 2, and some detailed steps were optimized for the scale-up manufacture as in the following:

a) The **HPH-VPG** was prepared by HPH, which followed the first research works published for VPG. Egg phosphatidylcholine 80% (EPC 80) was mixed with phosphate buffer for 2 h under magnetic stirring. The raw dispersions were homogenized at 700

bar for 10 cycles using a high-pressure homogenizer Micron Lab 40 as described earlier as ‘one-step' liposome preparation [7].

b) The **DAC-VPG** method has used the preparation parameter with 3500 rpm, 45 min as before [8,9], and consider the need for scale-up manufacture, the feeding amount was large to 5 g or 5 mL each time, and then, then all similar lipid content groups were mixed again by DAC to get the large volume samples before measurements.

c) The **MS-VPG** was prepared with the Triangular Stir Bars (40 mm X 14 mm, 442-0389, VWR), and was mixed the EPC 80 with phosphate buffer for 2 hours under very powerful Magnetic stirrer.

d) The **Extruder-VPG** preparation method was following the methods of Natalie Deiringer and Michaela Breitsamer [6,10]. The EPC 80 and phosphate buffer were mixed 5 min before extruder. The parameters of the Three-Tec extruder were set as 300 rpm, 30 °C, and the diameter of the terminal nozzle is 0.4mm.

The 30, 40%, and 50% lipid content VPGs were prepared by each method.

6.2.2 The properties of VPGs manufactured by scale-up processes

The appearance of VPGs from different preparation methods has slight differences, especial the high lipid content VPG groups. The status fresh of prepared 50% lipid VPG from different preparation methods are presented in Figure 6-1.

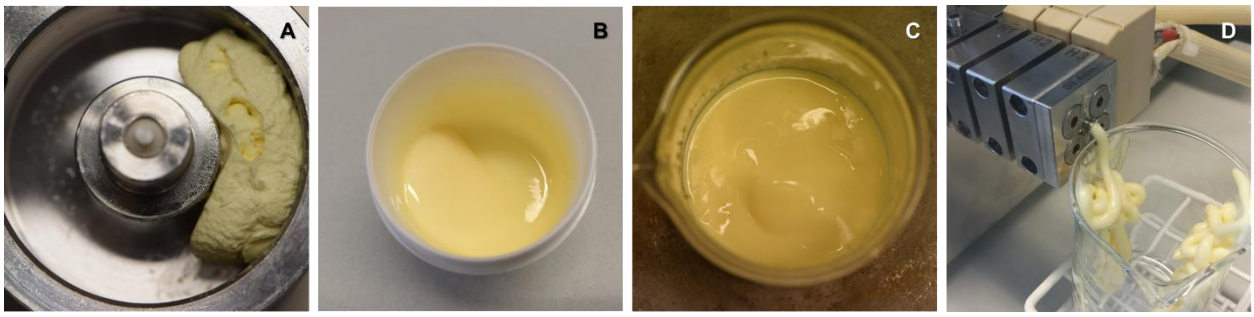

Figure 6-1 The 50% lipid VPG preparation by different methods. (A) High-Pressure Homogenizer (HPH), (B) Dual Asymmetric Centrifugation (DAC), (C) Magnetic Stirring (MS), (D) Extruder (Extru).

According to the appearance of gels from different methods, the surface of HPH-VPG was rougher than other groups, and it was full with small visible holes (Figure 6-1, A), and on the other hand, the appearances of VPGs from DAC and MS were similar (Figure 6-1, B, C), and the Extru-VPG (Extruder) was more like a soft stick (Figure 6-1, D). The HPH, DAC, and MS groups were set to a batch size of 20 g or 20 mL once, and the MS

can prepare 40 mL VPG once (Figure 6-2). The Extru method could supports a continuous preparation for VPG formulations.

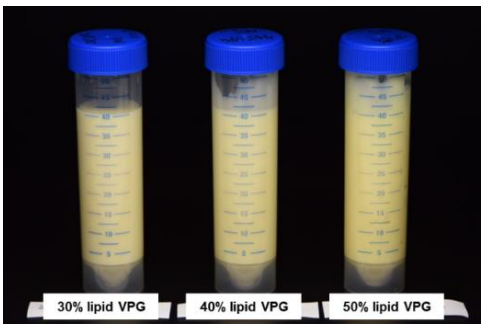

Figure 6-2 Scale-up manufacture of VPGs by MS

The Scanning Electron Microscopy (SEM) was used to analyze the surface status of the VPG (Figure 6-3). From these SEM pictures, the structures of VPG from different preparation methods appeared different, especially the VPG from DAC showed a “multi-balls” structure. And the structures of MS and Extruder methods were similar.

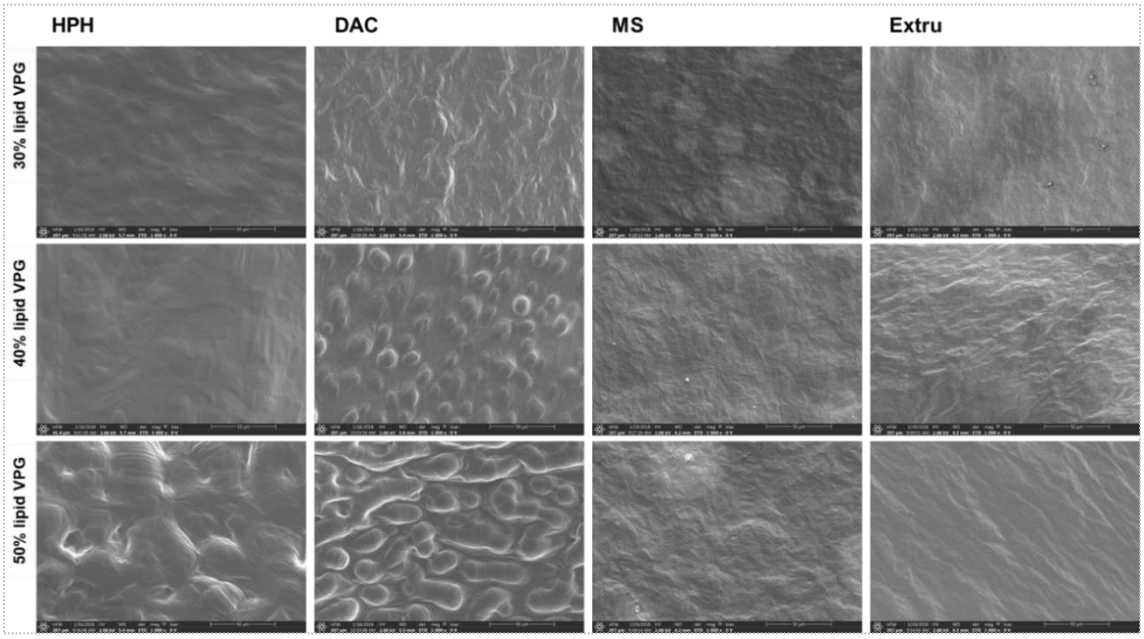

Figure 6-3 The surface structure of VPGs from different manufacture methods

The energy-dispersive X-ray spectroscopy (EDX) was also done in parallel with SEM, and the maps of different element distributions were acquired (Figure 6-4). From the co-localization of different elements (P, Na, O) in Figure 6-4, the P element distribution was very even in every group, but the Na and O elements were slightly different. The PBS distributions during the DAC operation were inhomogeneous (Figure 6-5), especially for the 50% lipid VPG, indicating that the DAC method may reach its limit at very high lipid concentrations. All the other methods use forced mixing by moving devices or tools,

whereas DAC uses free flowing mixing, and more investigation could be done in future research.

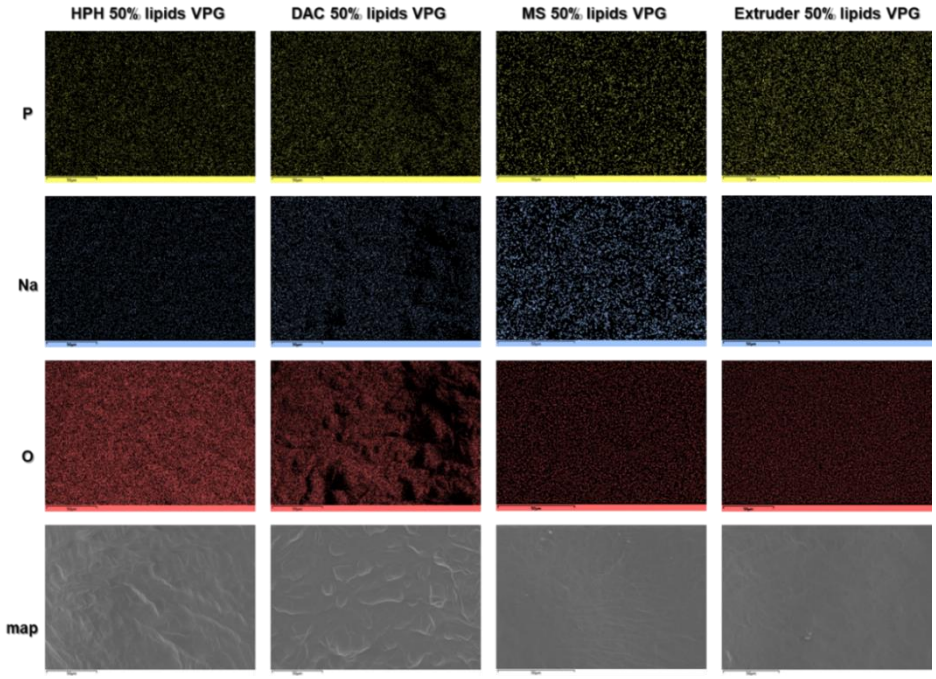

Figure 6-4 EDX analysis of the element distribution on the surface of 50% lipids VPGs from different manufacture methods

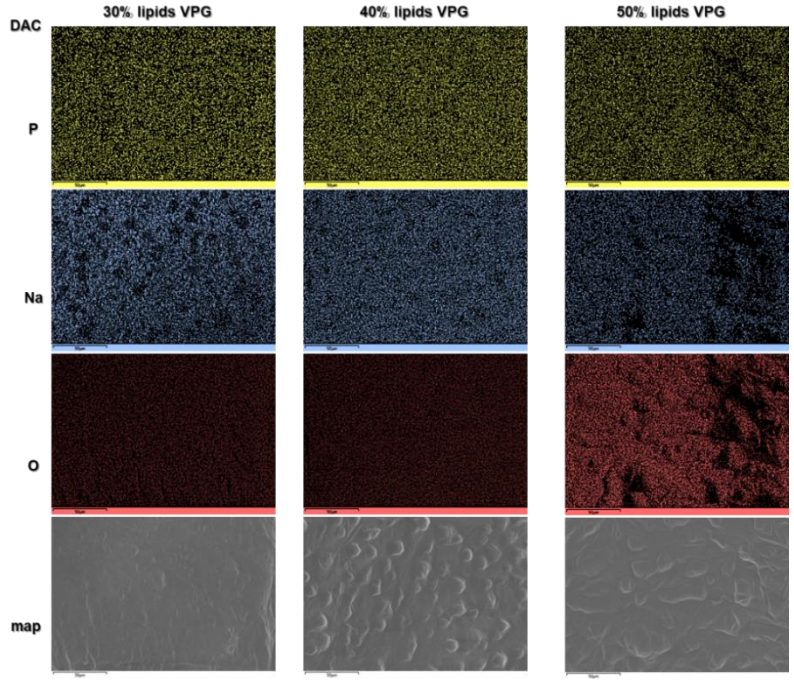

Figure 6-5 EDX analysis of the surface structure of different lipid content VPGs from DAC preparation.

6.2.3 The storage study of VPGs from scale-up manufacture by different methods

The VPGs from different preparation methods were put into the sample vials (both in polymethyl methacrylate cuvettes and glass vials) purged and sealed with Nitrogen gas. All samples were put into 4°C for storage study. The measurement time points were fresh, 1 month, and 3 months (Figure 6-6).

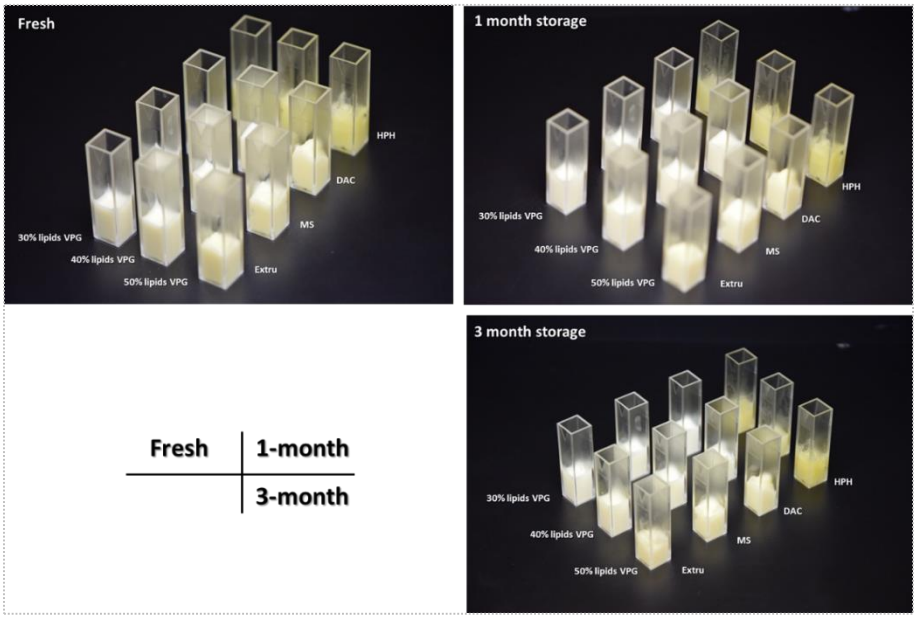

Figure 6-6 The general situations of VPGs storage experiments.

In this study, the HPH-VPG was different compared to the other groups, the color is more yellow, and already after the 1-month storage, a liquid and solid separation is visible in the 30% lipids VPG groups (Figure 6-7, 1-month storage). All other groups were very similar by observation (both plastic and glass vials). Maybe, the high pressure (force) of HPH method changes the micro-vesicle structure of VPG. After 3 months' storage, the liquid and lipid separation were more obvious in the 30% lipid HPH-VPG group, and other groups were still stable from appearance (Figure 6-7, 3-month storage).

Figure 6-7 The appearance of VPGs from different preparation methods after scale-up manufacture during the storage study. The 30%, 40%, and 50% lipid content VPGs were prepared by different methods, and stored for 1 month, and 3 months.

These samples were also analyzed by Rheometer (Viscosity), Texture Analyzer (TA), and Thermogravimetric analysis (TGA), and the results can be found in Figure 6-8

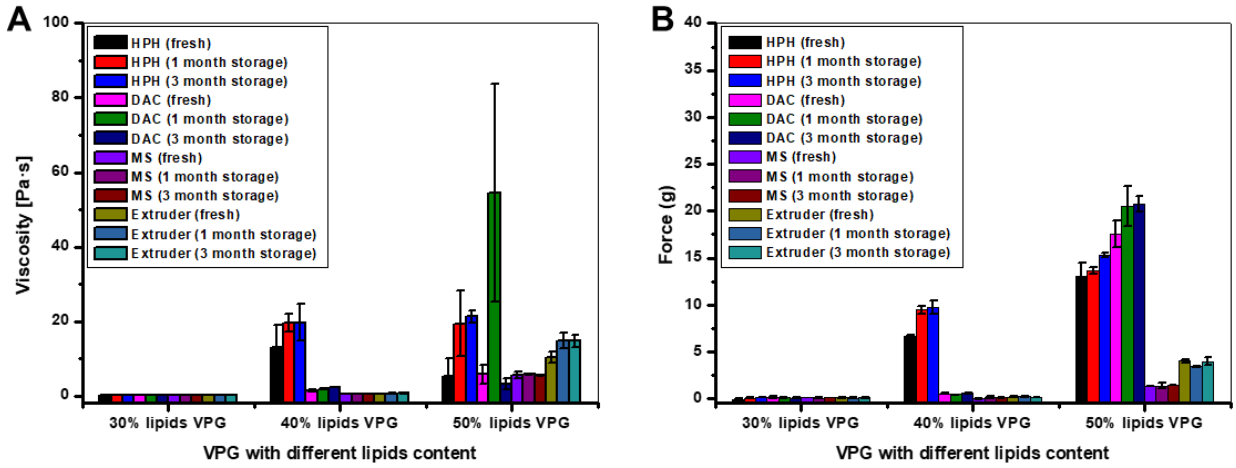

Figure 6-8 The viscosity and texture analysis of VPG formulations from different manufacturing methods for scale-up manufacture in storage study. (A) The viscosity measurements with Rheometer, (B) The strength measurements with Texture Analyzer.

According to viscosity measurement results, the lipid content was the main factor for the VPG viscosity, the viscosity was expectedly higher in the higher lipid content groups. At the same time, the 30% lipid VPGs from different methods were all similarly low in viscosity.

In the Texture Analyser measurement (Figure 6-8 B), there was also no significant difference in 30% lipid VPGs by different preparation methods. The 40% lipid VPGs from HPH methods had higher strength than all other groups in all time points. In the 50% lipid VPGs, surprisingly DAC-VPGs showed even higher mechanical strength force than HPH-VPGs.

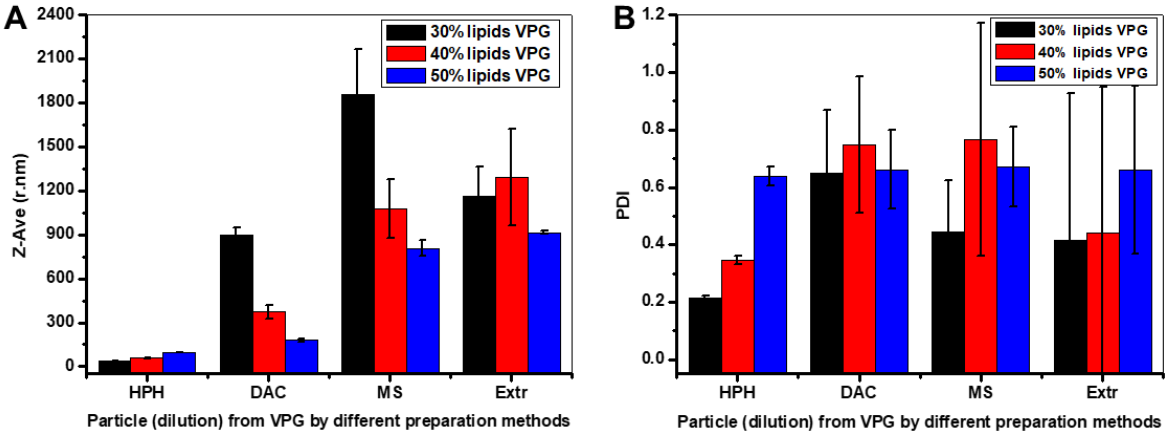

Figure 6-9 Particle analysis by 10000 times dilution by PBS after 3 months' storage.

The VPGs was diluted by buffer after 3 months' storage for the particles size analysis (Figure 6-9). The particles size of HPH-VPG was smaller than other group, and the particles size from MS and Extru methods were larger. The possible reason is the

shearing force in the HPH preparation is the strongest, which could homogenize any small particles in the formulation.

The Thermogravimetric analysis (TGA) was used for VPG microstructure analysis (Figure 6-10).

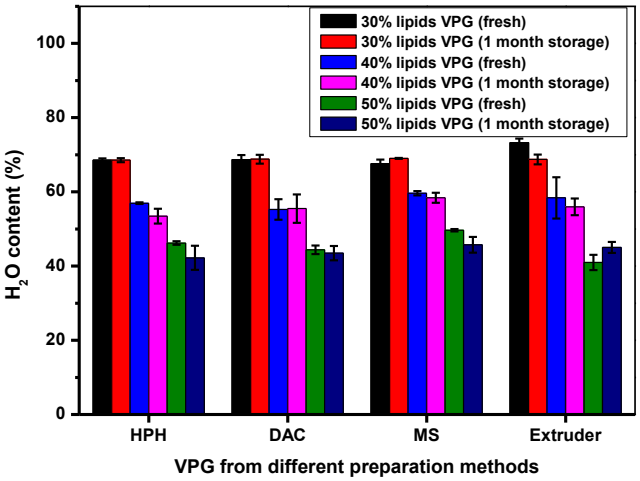

Figure 6-10 Thermogravimetric analysis (TGA) for VPG scale-up manufacture by different methods.

From TGA measurement, the water content was very similar for all process technologies and did not change much after 1 moth storage. On the other hand, the TGA could not separate the VPGs from different preparation methods for scale-up manufacture, which either means, that water is equally strong bound in all samples, and TGA cannot differentiate good enough.

6.3 Conclusion

Four methods for scale-up manufacture of VPGs were studied, and the advantages and disadvantages of those methods were general compared. The shearing force during mixing was the key physical factor of VPG manufacture, which brought different microstructure and other properties to the VPG formulations. The temperature control is another important point for the scale-up manufacture of bio-macromolecules. According to the performance of VPGs form these methods, we summarize a comparison of these methods in Table 6-1 and Figure 6-11.

	Ease of Operation	Gel Viscosity	Gel Strength	Homogeneity of VPG	Stability of VPG	Scale-up Capability
HPH		High				
DAC		Medium				
MS		Low				
Extruder		Low				

very good | good | satisfactory | insufficient | not suitable

Table 6-1 The overview of different methods for VPG scale-up manufacture.

* HPH: High-pressure homogenization, DAC: Dual asymmetric centrifugation, MS: Magnetic stirring, Extruder: Extrusion.
** There still no final conclusion about the evaluation about the viscosity, whether high or low value are better for the application.

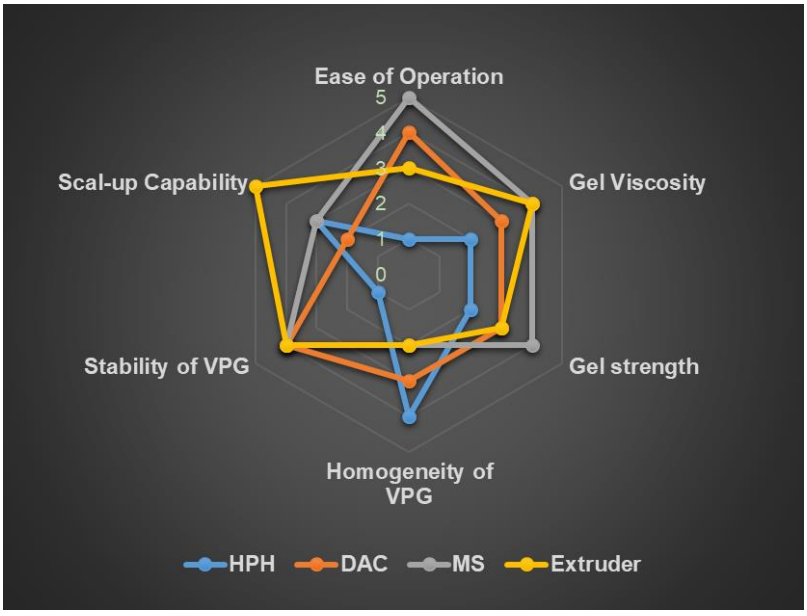

Figure 6-11 Comparison of different methods for VPG scale-up manufacture.

The HPH (High-Pressure Homogenization) was the first method for VPG preparation, but cannot be rated the preferred method for semi-solid formulation, because the shearing force was too strong, and too much heat was brought into the samples, which brought a separation of aqueous and lipid part after storage. On the other hand, the chamber volume was also a limit for large volume samples preparation. The scale-up preparation for low lipid content was well possible, but the operation was very difficult during the high lipid content semi-solid sample preparation.

Second, DAC could support good quality VPGs for biopharmaceutical research. DAC could mix samples up to 17 Kg in theory, but it was not suited for the high lipid content formulation scale-up preparation. The possible reason was that the shearing force was not strong enough in the high viscosity sample preparation, and the heat in the samples could not be cooled down. Different sample containers with better mixing function or a stronger cooling system are needed for further scale-up. On the other hand, during the first VPG preparation with DAC by Massing etc., the glass beads were added into for enhancing the mixing. The beads were removed to improve the simplicity of the process, and it working well during previous VPG preparation, but it could be considered into added back for high lipid content group (e.g. 50% lipid VPG) preparation with DAC for better scale up.

Third, the MS (Magnetic stirring) and Extruder had better performance in the scale-up manufacture, in which the continuous shearing force was more suitable for the large volume VPG preparation. These two methods could also support better temperature control condition in theory. The MS leads always to very low viscosities and it is not yet clear if this a benefit during further using (like drug loading, injection, and release performance, etc.). But the MS or other Stirring methods support an easy way to increase the preparation volume without special equipment. The extruder could support the continuous preparation for VPG formulation, and these methods could be brought into scale-up manufacture in the future for application. More factors should be investigated for scale-up manufacture, the Stirring (Magnetic or other stirring methods) and Extruder could be considered first.

6.4 Reference

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Chapter 7 Final Summary and Outlook

During the last decades, biotechnology and biomedicine are progressing with each passing day. Lots of new biomolecules were developed for new treatment methods, and more and more new advanced formulations were designed for the drug delivery. VPG is a semi-solid depot formulation with a very good capability for retarded protein drug delivery. This dissertation is focused on protein loaded VPG formulations, which are highly concentrated phospholipid dispersions, and have long-term sustained release performance. The research tries to develop a better method for protein loaded VPGs extraction and analytics, to develop better *in vitro* release test models for VPG formulations, to investigate good methods for long-term storage of protein loaded VPG formulations, and the scale-up of VPG manufacturing processes and pertaining to long-term stability studies on VPGs.

Chapter 1 is the general introduction of this dissertation, which includes the development background of protein drugs, an introduction into novel protein drug delivery systems, the history of VPG formulations, preparation methods for VPG formulations, the purification, and analysis of protein loaded VPG formulations, the *in vitro* erosion and release test models, and the long-term storage methods study. The AIM of this thesis is also stated in this part. **Chapter 2** has a detail description of the general materials and reagent, and also gives a detailed introduction for the experimental methods in this research.

The previous published purification methods were not suitable for the protein active analysis from VPGs, with the phospholipid concentrations up to 50%. In **Chapter 3**, a novel, easy and optimized purification and analysis method for antibody loaded VPG formulations was developed with the principle of physical adsorption. Several methods for protein extraction from VPGs were tested, and the residual concentrations of lipid and protein were measured. The 50CHPAPS-60LRA combination was selected because it delivered the best ratio of protein recovery to lipid contamination. The protein structural stability, chemical stability, and biological affinity were measured after extraction, which demonstrated that the VPG preparation and extraction was gentle enough for the antibody. With this method in place, more investigations could be carried out for different types of bio-macromolecules in drug loaded high phospholipid formulations. This extraction and analysis method makes the protein bioactivity analysis in high lipid content formulations easier and clearer.

Four different *in vitro* release models have been tested and evaluated for VPG formulations in **Chapter 4**. These release test models have presented different properties, each with advantages and disadvantages for *in vitro* VPGs investigation. FITC-Dextran loaded VPGs were tested as a surrogate for protein loaded formulations in those release test models. The release duration of the FTC w/o membrane model (Flow-Through Cell without membrane) was several weeks, and large particles like liposomes could also release from the formulations by erosion. The release rate of FTC with membrane model (Flow-Through Cell with membrane) was prolonged (several months), and the main release force is diffusion, and lipid particle could release from the sample chamber with an extremely low release rate. The AGG model (Agarose-Gel) could support both qualitative analysis and quantitative measurement. The diffusion force was also the main force during the release process in the AGG model, and the release rate was slowest. The erosion would not happen in AGG model measurement, and practically no lipid particles could release into the surrounding matrix. In the SFH model (Small Filter Holder) study, the release terms were about 3 to 6 weeks, and it was an easy and fast way to test the release behavior of VPG formulations. The SFH model allows certain erosion and diffusion from VPG local depot matrix in a rather controlled manner by using a coarse screen filter. In summary, the SFH model appears the most valuable one of the four models.

Small hydrophobic molecular drug (SAFit2) loaded VPGs were also tested both in an *in vitro* release measurements and in an *in vivo* PK study. The SFH could distinguish the different lipid content VPG formulations by different release behaviors, which is closer to the situation *in vivo*. During the *in vivo* PK study, the VPG formulations demonstrated the sustained release capability with higher relative bioavailability than an aqueous solution type formulation. Although the IVIVC study was not successful due to the quality of the data (Appendix 4), it was clearly demonstrated that the SFH model provides plausible data. With regard to the *in vivo* situation in the subcutaneous administration, where liposomes can slowly diffuse towards the next lymph node, a model that allows partly erosion from a depot sample without dispersing it immediately in a bulk fluid appears to be a reasonable concept. During this part of the research, the SFH model performance as a fast, easy and accurate model for *in vitro* VPG release investigations was good. More investigations could be done on the basis of SFH models for the VPG formulations in further research.

Chapter 5 focuses on long-term storage studies of monoclonal antibody (mAb) loaded VPG formulations. Several methods have been tested, which included storage of a freeze-dryed product and a frozen state product. A “fast freezing” freeze-drying method was developed for mAb loaded VPG formulations. This method supported good protein stability in long-term storage, which includes the structural, chemical, and bio-affinity stability after freeze-drying and after storage over 12 weeks’. Compared with the “wet” stored mAb loaded VPGs, the structure stabilities, chemical stabilities, and bio-affinity stabilities of protein were improved significantly. Unfortunately, the release rate from the VPGs decreased in the freeze-dried formulations over time, and more research must be done to improve this in the future. Second part of **Chapter 5** relates to the frozen state storage method, which also demonstrated feasibility to store the mAb loaded VPG formulations, with good protein stability protection. It also has the release rate decrease problems after storage after frozen, which should be solved before further application. It would be a low tech approach way to store protein loaded VPG formulations, and compared with freeze-drying it would be an easier option for clinical research project.

In **Chapter 6**, the methods to scale-up manufacture of VPGs were set up, and the advantages and disadvantages of different methods were compared. The shearing force during the gel (lipid and water) mixing was the key physical factor of VPG manufacture, and brings different microstructures and properties to the VPG formulations after different processes. The temperature control is another important point for the scale-up manufacture of bio-macromolecules in formulations. The HPH was historically the first method for VPG preparation, but did not perform well in our studies. DAC was a rather good method to manufacture a small amount of VPG formulations with better quality, and it has been demonstrated to be a good method to prepare the protein loaded VPGs. But the scale up is limited by definition and already at a small scale of 20 g problems with the high viscosity in 50% lipid VPG was visible. Magnetic stirring and the extruder had better performance in the scale-up manufacture, because the active shearing force was more suitable for the large volume VPG preparation. These two methods also allow better temperature control. Stirring (magnetic or other stirring methods) or extruder should be considered as preferred methods for future research according to the results of this work.

In this dissertation, different aspects of VPG formulation technology have been investigated, including the preparation, purification, *in vitro* release test methods, IVIVC, storage stability, and scale-up manufacture. A mAb was the core drug molecule in this

study. VPGs were demonstrated to be a good formulation for mAb encapsulation. In the future, different proteins (or other bio-macromolecular drugs) could be added into VPG formulations for sustained release depots on the basis of this dissertation. On the other hand, more *in vivo* pharmacokinetics and treatment experiments with animal models with VPG formulations are needed and should start in the near future.

Appendix

1. Sectional view of the AGG model

After the long-term release imaging experiment, the agarose gels were cut to test the sectional view of the gel. The drug diffusion range was the largest for aqueous solutions of FITC dextran, and the diffusion range decreased with increasing lipid content of the formulations (AP-1).

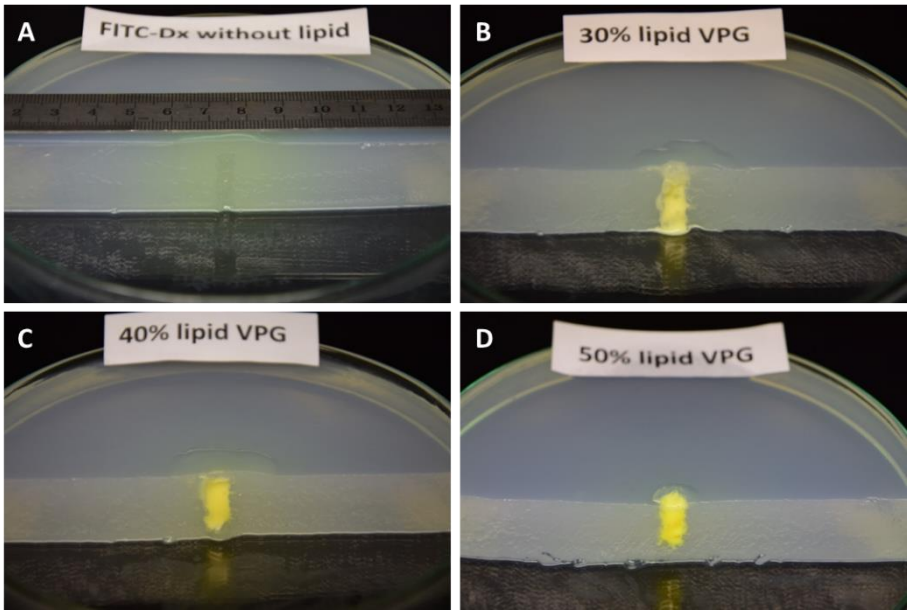

Figure AP-1 The sectional view of the AGG model after release imaging measurement (30 days point).

2. Particle analysis of release fraction in lyophilization study

The particle size of the released fractions during long-term storage was measured by DLS (Figure AP-2).

According to the DLS measurements, the release behavior at the initial time point (week-0) did not change for liquid and lyophilized mAb loaded 30% VPG (Figure AP-2, A-1, B-1, C-1, D-1), which means freeze-drying did not affect the sustained release capability of VPG. At the same time the slight erosion occurred between day 1 to day 3 (Figure AP-2, A-1, B-1), and the release behavior was very similar (Figure AP-2, C-1).

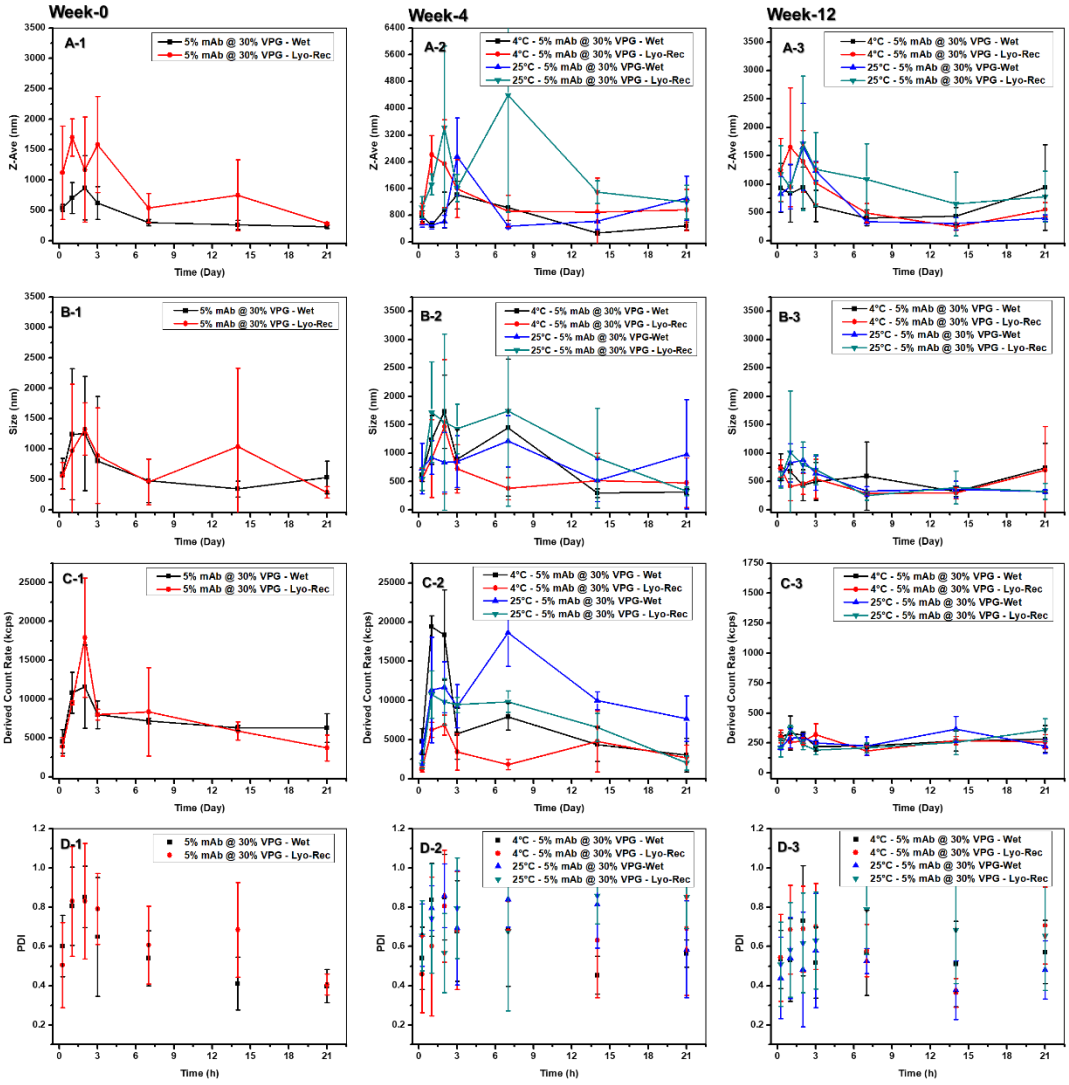

Figure AP-2 The release behavior of Wet and lyophilized mAb loaded VPGs. (A-1,2,3) The Z-average size of released particles. (B-1,2,3) The intensity mean value of released particles. (C-1,2,3) The Derived Count Rate (DCR) of released particles. (D-1,2,3) The PDI of released fraction particles.

After 4 weeks of storage, the release behaviors differed: the samples stored at 4°C were more similar to the initial time point (Figure AP-2, A-2, B-2, C-2). After 12 weeks of storage, the release rate decreased, the particles in the released fractions were smaller (Figure AP-2, A-3, B-3), and the release rate was slower (Figure AP-2, C-3). This could indicate a change in the microstructure after 3 months of storage, which should be investigated in a next step.

3. Monoclonal antibody (mAb) labeling

The monoclonal antibody (mAb) was used for the protein loaded VPG formulation study, and it was modified with a fluorescent marker as a preparation step for loaded VPG imaging tracking and quantification. The fluorescent signal was working well and the protein structure was stable.

The NHS-Fluorescein assay kit ® (*N*-Hydroxysuccinimide (NHS)-ester labeling reagents) are the simplest and most commonly used reagents for labeling proteins (detailed in Chapter 2). According to references, the antibody bulk solution contains the primary amines buffer, which is not compatible with a NHS-labeling reaction because they will react with the NHS-ester moiety. So a buffer exchange was needed before the NHS labeling. The buffer was exchanged to the 20mM PBS by dialysis. After the buffer exchanging, the 500 mg antibody (in 20 mM PBS, pH 7.4) was labeled with the NHS-Fluorescein (dissolved in DMSO) and then mixed for 1-hours at room temperature. After that, the reaction solution was put into the dialysis bag to remove the free fluorescein molecular. Then, after 3-days dialysis purification, the mAb-NHS was received. The absorbance of the sample was measured by the Spectrophotometer (280nm and 493nm), and the degree of labeling was 5.6 by the measurement and calculation.

The structure stability was tested by SDS-PAGE analysis, the reducing and no-reducing analysis were done. The mAb stock, mAb after buffer exchange, and modified antibody were analyzed (Figure AP-3). According to results, the molecular weight was slightly increased after labeling with fluorescence marker, which means the conjugation was successful.

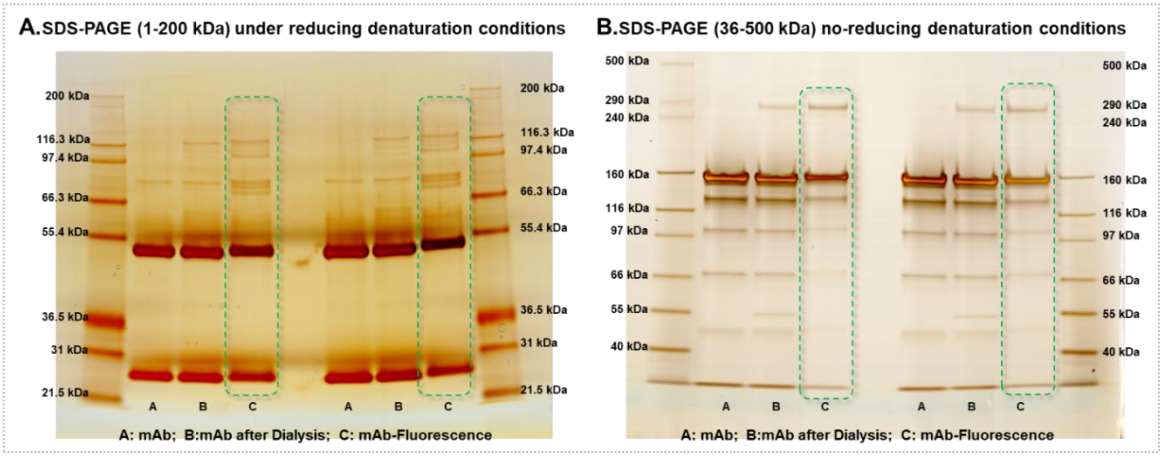

Figure AP-3 The SDS-PAGE of monoclonal antibody fluorescence labeling study. A, the SDS-PAGE under reducing denaturation conditions. B, SDS-PAGE no-reducing denaturation conditions.

The antibody stock solution, the antibody solution after buffer exchange, and the modified antibody were measured by SEC-HPLC (Figure AP-4).

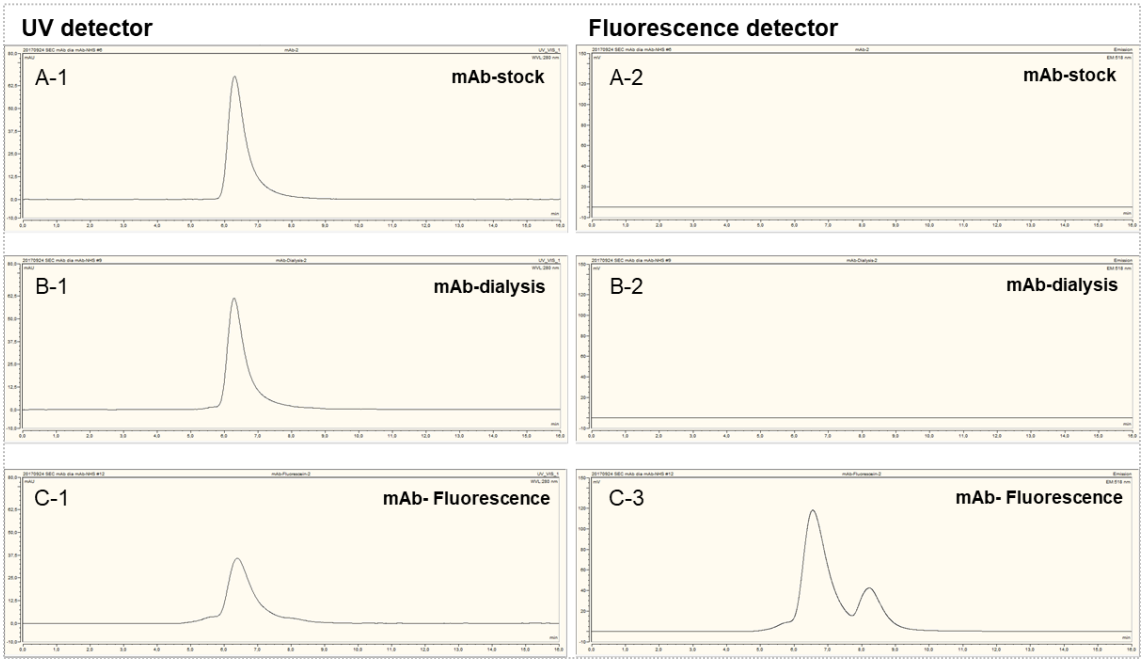

Figure AP-4 The chromatography of SE-HPLC for monoclonal antibody fluorescence labeling study. A-1, mAb stock in UV detector analysis. A-2, mAb stock in fluorescence detector. B-1, mAb after dialysis in UV detector analysis. B-2, mAb after dialysis in fluorescence detector. C-1, mAb-fluorescence in UV detector analysis. C-2, mAb-fluorescence in fluorescence detector.

According to the SEC-HPLC results, the molecular structure was stable enough, and at the same time, from the signal of fluorescence detector, the main peak time was the same with the UV detector, which means the fluorescence was labeling to the mAb molecules successfully. The fluorescence-labeled mAb could be used in the antibody loaded VPG formulations study in the future.

4. The *in vitro–in vivo* correlation (IVIVC) for the small molecule loaded VPGs

The *in vitro–in vivo* correlation is a predictive mathematical model describing the relationship between an *in vitro* property and a relevant *in vivo* response of formulations. The IVIVC calculation for SAFit2 loaded VPGs were did on Phoenix® IVIVC Toolkit™ by Dr. Bernd Wendt (Certara®). A “one-stage” IVIVC approach has been applied to a set of dissolution (*in vitro*) and *in vivo* data. A model was constructed that predicts plasma concentration as a function of absorption, which is itself a function of dissolution.

4.1 Modeling *in vitro* data

The Weibull model is a widely used model to describe dissolution data. In first step: The cumulative release data is expressed as percentages. For modeling purposes the percentages were converted to fraction dissolved (FDISS). The second step: Fitting a Weibull model to the data.

i. Weibull model [16]: $y(t)=Finf\ (1 - \exp[-(t/MDT)^{b}])$

ii. Results:

a. Plots: The Weibull model describes the profiles accurately in Figure AP-5.

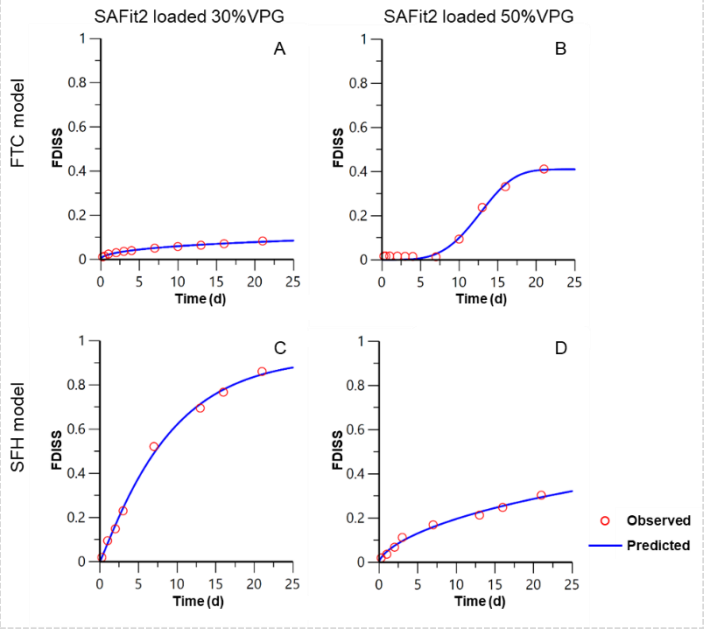

Figure AP-5 The description plots for profiles accurately by Weibull model. (A) The SAFit2 loaded 30%VPG in FTC release model. (B) The SAFit2 loaded 50%VPG in FTC release model. (C) The SAFit2 loaded 30%VPG in SFH model, (D) The SAFit2 loaded 50%VPG in SFH model.

b. Parameters (Table AP-1): The shape parameter B varies between 0.5 and 4 reflecting the differences of the individual curvatures. The curvature for 50%VPG in FTC with a B value of 4 is significantly different from the other 3 formulations with B values below and up to 1. Mean dissolution times (MDT) also varies across the formulations with values between 9 and 74 days. Fraction at infinity (FINF) also varies with values closer to 1 from the SFH method and values less than 0.5 for the FTC method.

Formulation	B	MDT	FINF
30%VPG_in_SFH	1.08	9.09	0.93
50%VPG_in_SFH	0.65	73.76	0.83
30%VPG_in_FTC	0.50	69.99	0.19
50%VPG_in_FTC	3.97	13.80	0.41

Table AP-1 The shape parameter of *in vitro* fit

4.2 Modeling IVIVC

a. The 1-compartment model for the solution data is taken as reference to predict the *in vivo* profiles of the patch data. The 1-compartment model is changed to an IVIVC model by replacing the differential equation for absorption with a differential equation of the first derivative of the Weibull function. The Weibull parameters of our dissolution model are used in combination with the PK parameters of our 1-compartment model. All those parameters are frozen for the fit of the model to the patch in vivo data. Only one parameter is estimated: T_{scale} – a scaling parameter to reflect the different times of *in vitro* and *in vivo* data.

i. Plots: For all 4 models the fit to the *in vivo* data is not accurate. The worst fit is for 30% VPG formulation and the FTC method and the best fit is for the two models of the SFH method. However, all 4 models are not able to fit the second peak on day 7.

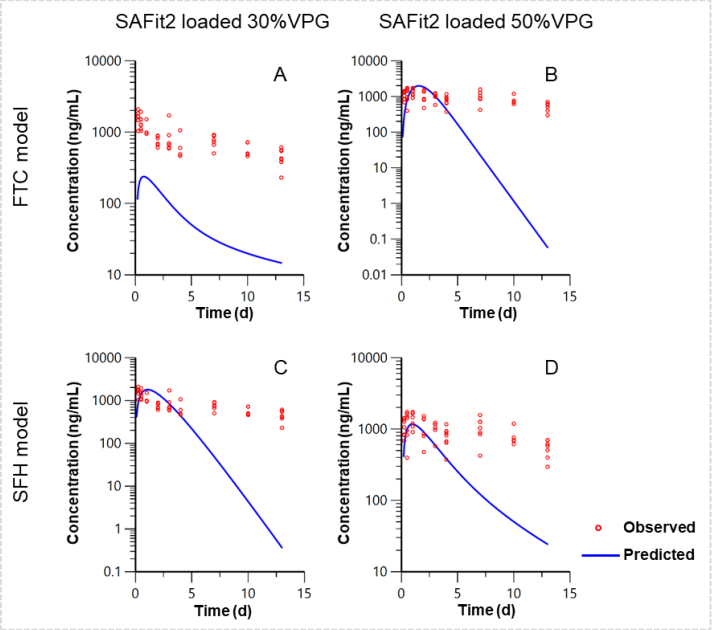

Figure AP-6 The plots of IVIVC fit. (A) The SAFit2 loaded 30%VPG in FTC release model. (B) The SAFit2 loaded 50%VPG in FTC release model. (C) The SAFit2 loaded 30%VPG in SFH model, (D) The SAFit2 loaded 50%VPG in SFH model.

ii. Model parameters: T_{scale} was the only parameter estimated by fitting the model to the data: *In vivo* time is shorter than *in vitro* time, which in itself is unusual, but also the values show a large difference between *in vitro* and *in vivo* times with up to 1-2 log units.

Source	Parameter	Estimate
30%VPG_in_SFH	T_{scale}	0.1257
50%VPG_in_SFH	T_{scale}	0.01426
30%VPG_in_FTC	T_{scale}	0.01261
50%VPG_in_FTC	T_{scale}	0.09543

Table AP-2 T_{scale} parameter by fitting the model to the data

iii. Model diagnostics: The -2 Loglikelihood, the Aikaike or Bayesian Information criterion is used to rank the fits, the lower value of those criterions indicates the better fit.

Source	-2LL	AIC	BIC	nParm	nSub
30%VPG_in_SFH	**807.96**	**811.96**	**815.86**	2.00	7.00
50%VPG_in_SFH	**948.00**	**952.00**	**956.16**	2.00	8.00
30%VPG_in_FTC	**862.87**	**866.87**	**870.77**	2.00	7.00
50%VPG_in_FTC	**940.87**	**944.87**	**949.02**	2.00	8.00

Table AP-3 Model diagnostics for IVIVC fitting. -2LL: –2 Loglikelihood. AIC: Akaike Information Criterion for each model run. BIC: Bayesian Information Criterion for each model run. nParm: Number of formulations. nSub: Number of subjects [16].

iv. Model validation: PK parameters Cmax and AUClast were calculated for observed and predicted profiles. The percentage error between observed and predicted profiles

was determined. The lowest errors (absolute value) are those from the 30% VPG formulation and the SFH method.

	Parameter					
	AUClast			Cmax		
Source	CObs	C	**PE%**	CObs	C	**PE%**
30%VPG_in_SFH	9597.50	5241.18	**-45.39**	1601.35	1792.48	**11.94**
50%VPG_in_SFH	11708.23	4097.67	**-65.00**	1340.36	1168.88	**-12.79**
30%VPG_in_FTC	9597.50	853.03	**-91.11**	1601.35	238.11	**-85.13**
50%VPG_in_FTC	11708.23	5244.72	**-55.20**	1340.36	1972.88	**47.19**

Table AP-4 Model validation for IVIVC fitting. AUClast: Area under the curve from the time of dosing to the time of the last measurable (positive) concentration (Tlast). Cmax: Maximum observed concentration, occurring at time Tmax, as defined above. CObs: The continuous observations of drug concentration in the blood. C: The Predicted of drug concentration by *in vitro* test. %PE (prediction error) = (Predicted-Observed) / Observed x 100% [16].

4.3 Summary for IVIVC

According to the correlation results between the *in vitro* release test with *in vivo* PK study, the release test models for VPGs formulation could be evaluated. Dissolution models and a PK compartmental model were developed for 2 different formulations (30% VPG, 50% VPG) combined with 2 different dissolution methods (SFH model, FTC model). Accurate fits could be achieved for all 4 dissolution profiles using the Weibull model. Resulting parameters from the dissolution fits vary widely, in terms of mean dissolution time (MDT), fraction dissolved at infinity (FINF) as well as shape (parameter B). This model was combined with dissolution model to form a one-stage IVIVC model. In general, the fits were not accurate. The FTC model was performed not good in both 30% and 50% lipid VPG formulation test correlation, and at the same time, the SFH model has the lowest errors during the SAFit2 loaded 30% VPG formulation for IVIVC, which demonstrated the SFH model was more suitable for this formulation *in vitro* release behavior investigation.

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