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Research paper

Do interactions between protein and phospholipids influence the release behavior from lipid-based exenatide depot systems?

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ABSTRACT

The release mechanism for proteins and peptides from vesicular phospholipid gels (VPGs) is very complex. Drug release proceeds via a combination of erosion of the gel and diffusion of the drug out of it. This diffusion can be retarded by a slow permeation of the drug across the lipid bilayers in the gel as well as by its direct binding or adsorption to the lipid bilayers. Finally, the viscosity and homogeneity of the formulation may affect the release behavior. So far a direct correlation between one of these parameters and the release kinetics is not possible.

In the present study, we aimed to investigate the contribution of drug-membrane interactions to the release kinetics of exenatide from differently composed VPGs (POPC, POPG and mixtures of both). To this end, in vitro release of exenatide as well as in vitro release of the phospholipids was monitored. Binding affinities were determined by microscale thermophoresis (MST).

The sustained release behavior of exenatide could not simply be correlated to high viscosity of the VPG formulation. Release of exenatide from VPGs of anionic membranes containing POPG proceeded with a half-life of the order of 5 days and it seems to be controlled by the erosion of the gel. Its rate is unaffected by the initial pH inside the gel, independently of the strong impact of pH on exenatide binding to the membrane. At pH 4.5, exenatide is cationic and binds to membranes containing anionic POPG with a high affinity ($K_d \approx 10\text{–}30\ \mu M$).

No high affinity membrane binding of exenatide is detected in this at pH 7.4, where exenatide is anionic, and to zwitterionic membranes composed of POPC. Exenatide release from the latter has a significantly longer half-life of 30 to 55 days. That means, these VPGs are much more resistant to erosion and show a very slow diffusional release. In this case, diffusion should be slowed down by the barrier function of the membranes rather than membrane affinity.

In conclusion, erosion of the VPG matrix and membrane permeability of the drug are the major parameters influencing the release of exenatide from VPGs of POPC-POPG, whereas drug binding to the membranes had a minor effect only.

1. Introduction

Vesicular phospholipid gels (VPGs) are semisolid drug formulations which are composed of phospholipids and a simple aqueous phase only [1,2]. They are prepared by elegant one step manufacturing techniques like high pressure homogenization (HPH) or dual asymmetric centrifugation (DAC). Their capability to release drugs and especially proteins and peptides with a slow release rate, combined with their easy and cheap manufacturing and their high biocompatibility makes them very promising drug delivery systems [3–6]. Next to their application for large molecules like proteins, they have also been used for the sustained delivery of small molecules [7–12]. The administration via

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Abbreviations: DAC, dual asymmetric centrifugation; DLS, dynamic light scattering; FT-IR, Fourier-transform infrared spectroscopy; HPH, high pressure homogenization; HPW, highly purified water; MCA, micro capillary array; MST, microscale thermophoresis; MWCO, molecular weight cut-off; PBS, phosphate buffered saline; PC, phosphatidylcholine; PDI, polydispersity index; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1′-*rac*-glycerol); QCM, quartz crystal microbalance; RP-HPLC, reverse phase high performance liquid chromatography; SLI, solid lipid implant; SPR, surface plasmon resonance; TFA, trifluoroacetic acid; VPG, Vesicular Phospholipid Gel

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needle-free injection facilitates the versatile application of VPGs [13].

The release behavior of VPG is dominated by a combination of an erosion controlled and an diffusion controlled process [5]. Tardi et al. found that these two processes occur at the same time. VPGs with up to 30% (m/m) phospholipids disintegrate fast. VPGs with 35–40% (m/m) phospholipids show erosion controlled release behavior and VPGs with a phospholipid amount above 45% (m/m) show rather diffusion controlled release kinetics. However, there seems to be more behind this release process. Depending on the encapsulated drug substance and the phospholipid composition, changes in release behavior have been observed [14–16]. Possible interactions between the encapsulated protein or peptide and the phospholipids may be responsible for the different sustained release profiles.

In this context, Neuhofer et al. addressed the interactions between interferon β-1b and the triglycerides H12 and D118, but also different phospholipids [14]. Additionally, he found that the extraction of native interferon β-1b from VPG was not possible and concluded that interactions were responsible for this phenomenon. PEGylation reduced the interaction effects but could not completely avoid it. He also found that the presence of a protein drug affected the mechanical properties of the VPG matrix and concluded that interactions may be responsible. To this end, he used a Langmuir film balance and a quartz crystal microbalance (QCM) to investigate different models like liposomes or monolayers. But no consistent correlation between the different methods and no prediction of the release behavior could be established.

Even et al. investigated the interactions of peptides with different charge and hydrophobicity with lipids including D114, soy lecithin, and cholesterol by adsorption studies [16]. It was shown that the size, hydrophobicity, or charge of encapsulated proteins or peptides are not alone responsible for the sustained release from VPGs. The release of a small peptide from VPGs was, against all expectations, slow and incomplete, whereas a much bigger protein was released fast in comparison. Additionally, no direct relationship between hydrophobicity of the molecule and release rate could be identified.

Further, it was shown that the high dispersity of VPG produced by DAC or HPH, including small vesicles and multi-lamellar structures, is not necessarily needed to obtain a sustained release behavior [17]. Zhang et al. showed in vitro and in vivo sustained release of exenatide from VPGs after preparation by magnetic stirring.

These indications in literature brought us to the hypothesis that sustained release of proteins or peptides from lipid-based depots could be controlled by interactions between the molecules, more specifically by a binding of the peptides to the phospholipid layers. The possibility to tune the release behavior by using these interactions motivated us to further investigate and characterize the interactions between proteins and phospholipids in this context.

Interactions between proteins and phospholipids have been studied extensively in the past with various methods (SPR, MST, ITC, Flotation assays etc.) [18]. However in such studies, the focus was mainly on interactions of proteins with cellular membranes [18–21]. Only few studies on the release behavior of lipid-based depots have been conducted so far and in many cases, the focus was on solid lipid implants (SLI) as drug delivery system and triglycerides as their main components [14,16,22].

In this study, we aimed to investigate the interactions between exenatide, a pharmaceutically highly relevant, relatively small peptide with a defined secondary structure and phospholipids with microscale thermophoresis (MST) measurements and correlate our findings to the release behavior from VPGs.

2. Material and methods

2.1. Material

Exenatide acetate was purchased from Chemos GmbH&Co.KG (Regenstauf, Germany) and purified by dialysis (MWCO 5 kDa) into highly purified water (HPW, Purelab Plus, USF Elga, Germany) before freeze-drying of the solution for storage. An exenatide stock solution was freshly prepared in 50 mM sodium acetate buffer pH 4.5 or 20 mM phosphate buffered saline (PBS) pH 7.4 with a concentration of 200 µM. Dilutions thereof were used for interaction measurements.

All phospholipids (POPC, POPG) were purchased from Lipoid GmbH (Ludwigshafen am Rhein, Germany).

2.2. Exenatide characterization

A Zetasizer nano ZS (Malvern Instruments, UK) was used for determination of the zetapotential of exenatide. Zetapotential of an exenatide solution with a concentration of 5 mg/ml was measured in HPW after adjustment of the pH value with 1 M hydrochloric acid or 1 M sodium hydroxide.

FT-IR measurements were used to investigate the secondary structure of exenatide with changing pH value. A Tensor 27 spectrometer (Bruker Optics, Ettlingen, Germany) using a BioATR cell II (Harrick, New York, USA) was used to record FT-IR spectra. For each measurement, 30 µl sample with an exenatide concentration of 5 mg/ml was pipetted into the cell. Measurements were controlled using OPUS 7.5 and transmission spectra were truncated from 1350 cm^{-1} to 1750 cm^{-1}. Further spectra were edited by vector normalization and second derivatives were calculated using a 17-point smoothing function.

2.3. Preparation of vesicular phospholipid gel

VPGs with a phospholipid amount of 40% (m/m) were prepared by dual asymmetric centrifugation (Speedmixer™ DAC 150 FVZ, Hauschild GmbH & Co KG, Hamm, Germany) as described previously [3,6,13]. Four different phospholipid combinations were prepared: POPC and POPG were used as sole phospholipids; additionally, mixtures of POPC and POPG in a ratio of 7/3 and 1/1 were prepared. Phospholipids were accurately weighed in the required amounts before exenatide solution was added until a final exenatide concentration of 0.5 mg/g was reached in the VPG.

2.4. VPG characterization

Rheology measurements were performed to characterize the VPG formulations [3].

The viscosity of the VPG at a shearing rate of 32.9 s^{-1} was determined by a rotational rheometer (Physica MCR 100, Anton Paar GmbH, Ostfildern, Germany) with a plate-plate geometry. All measurements were performed at 25 °C and the shear rate was set to 10–100 s^{-1}. Approximately 0.2 g VPG was used per measurement.

2.5. In vitro release of exenatide from VPG

For the in vitro release studies, 200 mg of VPG were carefully overlaid with 1 ml 20 mM PBS buffer pH 7.4 in 2 ml centrifugation tubes. Then, all samples were incubated at 37 °C and 30 rpm in a vertical incubation shaker (Certomat incubation Shaker, Sartorius AG, Germany) in upright position. Release experiments were carried out by complete buffer exchange [13,16].

The concentration of exenatide was determined using a reverse phase high pressure liquid chromatography (RP-HPLC). A Jupiter 5 µm C18 300A column (Phenomenex, USA) was used for quantification of the peptide. Mobile phase A was composed of 10% acetonitrile, HPW and 0.1% TFA and mobile phase B was composed of acetonitrile and 0.1% TFA. Table 1S shows the multi-step-gradient which was used for the quantification of exenatide with a flow of 0.5 ml/min. Column temperature was set to 55 °C and the retention time of exenatide was 12 min. Exenatide was extracted by 1:1 dilution with ethanol, heating for five minutes at 60 °C, followed by 20 min of cooling at 2–4 °C and five minutes centrifugation. The resulting supernatant was taken for quantification.

2.6. Characterization of VPG erosion during in vitro release

The erosion behavior of the differently composed VPGs was characterized in parallel to the in vitro release of exenatide. One way of quantifying erosion was in terms of sample turbidity which, in turn, was expressed on the basis of optical density.

All samples were diluted as needed (1:4 or 1:10) with highly purified water and the absorbance at a wavelength of 600 nm was determined using a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany). 300 µl of diluted sample were pipetted into U-bottom 96-well-plates (MICROLON® 200, greiner bio-one, Frickenhausen, Germany). Absorbance values after dilution were between 0.1 and 1.5 and optical densities of the undiluted samples were calculated.

Alternatively, erosion of the lipid matrix was determined by quantification of PC in the released fractions. A LabAssay™ Phospholipid (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany) was used for this purpose. Samples were prepared following the supplier's instructions and measured at a wavelength of 600 nm using a FLUOstar Omega plate reader (BMG LABTECH, Ortenberg, Germany). In this assay, phospholipids are hydrolyzed by phospholipase D and the liberated choline is then oxidized by choline oxidase to betaine [23]. Thereby, hydrogen peroxide is produced which, in turn, initiates the coupling of 4-aminoantipyrine and N-ethyl-N-(2-hydroxy-3-sulfopropyl)-3,5-dimethoxyaniline sodium salt (DAOS) to a blue chromophore by oxidation catalyzed by peroxidase.

2.7. Liposome preparation

Liposomes were prepared by thin film hydration method. The respective phospholipid was solved in chloroform (25 mg/ml), the accurate amount of phospholipid solution was mixed as needed using a vortexer and the solvent was removed under a constant stream of nitrogen while the glass vial was turned producing a thin phospholipid film on the wall of the glass vial. After that, the film was hydrated with 50 mM sodium acetate buffer pH 4.5, 10 mM Tris buffer pH 7.4 (containing 110 mM NaCl and 0.5 mM EDTA), or 20 mM PBS pH 7.4. Liposomes were extruded by a Lipex (Burnaby, Canada) extruder. Five extrusion cycles through a 200 nm polycarbonate membrane were followed by several extrusions through a 100 nm polycarbonate membrane to achieve homogeneous liposome dispersions. Size, PDI, and zeta potential were determined by dynamic light scattering (DLS) using a Zetasizer nano ZS (Malvern Instruments, UK).

The lipid concentration in the liposome dispersion was determined using a gravimetric determination [24].

2.8. Microscale thermophoresis

Microscale thermophoresis (MST) measurements were performed using a Monolith NT.LabelFree (NanoTemper Technologies GmbH, Germany) with MO.Control v1.5.3. Dilution series were prepared for each of the liposome dispersions (1.0 mM to 3.05E − 05 mM or 0.5 mM to 1.53E − 05 mM) following the instrument's instructions. Then exenatide stock solution was added. During the assay, the exenatide concentration was kept constant at 2.5 or 5 µM, respectively. All samples were either prepared in 50 mM sodium acetate buffer pH 4.5 containing 0.1% Pluronic F-127 or in 20 mM PBS pH 7.4 containing 0.1% Pluronic F-127.

Samples were loaded into coated glass capillaries (Monolith NT.LabelFree Capillaries, Zero Background, MST Premium Coated). Then, capillaries were placed on a tray and inserted into the instrument before fluorescent scans were performed to determine the position of the capillaries. After that, 16 subsequent thermophoresis measurements (LED 20%, IR laser: Medium-Power) were performed. Ambient temperature of the instrument was set to 23 °C. For each ligand, a pre-test (with buffer, liposome-dispersion, and exenatide) was performed to qualify the sample for measurement before the binding affinity test, using the parameters determined in the pre-test. Dissociation constants were calculated after the measurement using NanoTemper MO.Affinitiy Analysis v2.2.7 software.

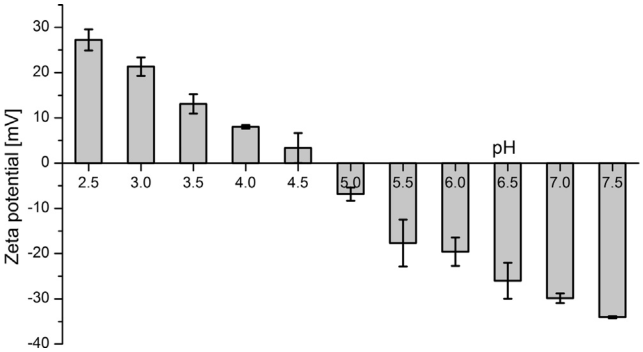

Fig. 1. Zeta potential of exenatide with increasing pH value. (Mean ± SD, n = 3).

3. Results

3.1. Exenatide characterization and encapsulation in VPGs

Determination of the zeta potential of exenatide over a broad range of pH values showed that exenatide turns from positive to negative between pH 4.5 and pH 5.0 (see Fig. 1). This is in good accordance with the literature value for the isoelectric point of exenatide of 4.86 [25].

With FT-IR spectroscopy, the secondary structure of proteins and peptides can be analyzed. In order to get further information about the folding behavior of exenatide at different pH-values, FT-IR measurements were conducted. The measurements confirm the alpha-helical structure of exenatide, which is described in literature [26]. This alpha-helical structure is preserved over the complete pH range from 3.5 to 7.5 (see Fig. 2). No changes or shifts in amid I or amid II band were observed, indicating structural integrity of the protein over the complete pH range.

Exenatide was successfully encapsulated into VPG by direct encapsulation process using a DAC as described [6]. In comparison to other preparation methods, this technique is very gentle and has been used for the preparation of protein loaded VPG before [3]. Exenatide loaded VPG were prepared containing 40% (m/m) of phospholipid and

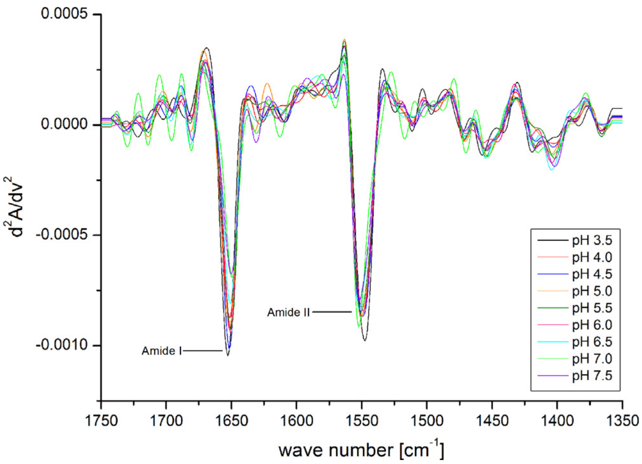

Fig. 2. FT-IR spectra of exenatide solution with increasing pH value.

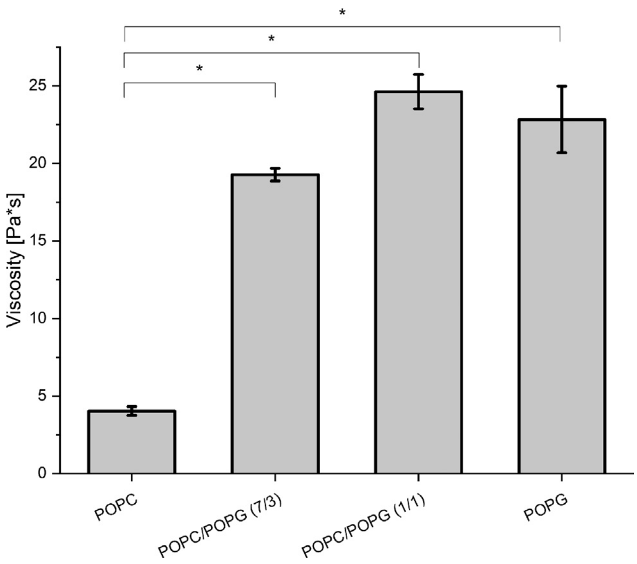

Fig. 3. Viscosity of VPG with a total phospholipid content of 40% (m/m) loaded with exenatide (0.5 mg/g). VPG was prepared with different phospholipid compositions. For comparison the viscosity at a shearing rate of 32.9 s^{-1} was taken. (Mean ± SD, n = 3, $p < 0.05$).

characterized by visual inspection and viscosity measurements.

Visual inspection exhibited a homogeneous appearance of the VPG without lipid agglomerates. Rheological behavior of the VPG was studied by rotational rheometry. The viscosity of the VPG was compared at a shearing rate of 32.9 s^1.

All formulations containing POPG showed a high viscosity (above 15 Pa s) (see Fig. 3). VPG formulations of POPC displayed lower viscosity (below 5 Pa s). The viscosity of the VPG was not influenced by the encapsulation of the peptide, i.e., placebo VPG showed similar values.

3.2. In vitro release of exenatide from VPG

Unlike Franz diffusion cells as commonly used in vitro release model for semi-solid drug depots, the centrifugation tube model chosen in this study takes into account erosion and diffusion of the VPG alike since no membrane is needed to separate the donor compartment from the acceptor side. The in vitro release of exenatide from VPGs was determined using formulations with pH 4.5 and with pH 7.4. In both cases, exenatide is released very slowly from VPG composed of POPC only, while a faster release is observed from formulations composed of POPC/POPG mixtures or the single POPG formulation. Complete exenatide release within 16 days was observed for the formulations composed of a mixture of POPC and POPG or the POPG only formulation, while VPG composed of POPC only released a maximum of 40% of the encapsulated drug within this period. No burst effect was observed for all tested formulations and encapsulation pH values.

At the same time, only slight differences between the two encapsulation pH values were observed. At an encapsulation pH 7.4 (see Fig. 4B), exenatide is released faster from the formulation containing POPC only in comparison to encapsulation at pH 4.5 (see Fig. 4A). Otherwise, the previously observed trend, a faster release from formulations containing POPG, was confirmed.

To determine if the differences in release speed were statistically significant, in vitro release data of each sample was fitted linearly (fit not shown) and the slopes of the obtained fits were compared with each other using a *t*-test. The differences in the slopes of the linear fits and thus the differences in release speed were found to be significant for all VPG compositions ($p < 0.05$).

The release kinetics were fitted by a model taking into account the possibility of two fractions A_1 and $1–A_1$, with different release rates k_1 and k_2:

$$c_{released} = c_{\max}\cdot[1 - A_1\cdot e^{k_1 t} - (1 - A_1)\cdot e^{k_2 t}] \tag{1}$$

The half-life of the drug fraction in its retained state, $t_{1/2}$, is then given by:

$$t_{1/2} = \frac{\ln 2}{k} \tag{2}$$

analogously for k_1 and k_2. The dashed lines in Fig. 4A and B show the fit lines.

A biphasic release behaviour was identified for the formulations containing POPG. One fraction (5–20%) being released with a half-life of less than 20 min and a second fraction (80–95%) being released with a half-life of 5 days. In contrast to that, formulations composed of POPC have a significantly longer half-life of 30–50 days (approximately 80%) with a small fraction (20%) being released with a half-life of 1–10 days.

3.3. Erosion behavior of VPGs during exenatide release

One possible mechanism of drug release from VPGs is the erosion of the lipid matrix. Optical density (OD) of the release fractions correlates to the erosion of the lipid matrix. When optical density is plotted cumulatively, erosion profiles for phospholipids are obtained, which are shown in Fig. 4C. Erosion of the VPG composed of POPC is rather slow in comparison to the three formulations containing POPG.

Fig. 4D shows the PC release profiles for the four formulations containing POPC. Slow PC release is observed for POPC-VPG while fast release is observed for the two mixtures with POPG. These results do correlate rather well with the release profiles found for exenatide. For formulations containing POPG, release of exenatide can be correlated linearly to the erosion of the VPG from the beginning. In contrast to that, for formulations composed of POPC a linear correlation between exenatide release and erosion can be found after a lag-phase of few days. This lag-phase may be a result of the release of loosely bound exenatide in the VPG which is released faster. After the lag-phase, erosion of the VPG composed of POPC sets in and controls the release behavior.

3.4. Interactions studies

When testing the in vitro release behavior of exenatide from VPG, a correlation between viscosity and release kinetics was expected in a way that highly viscous systems should release slowly and vice versa. However, since this expectation was not fulfilled, interactions between the encapsulated peptide and the phospholipids were investigated to see if these are responsible for the slow sustained release from VPG composed of POPC only.

Interaction studies were performed by MST measurements. Since MST has to be performed in free "solution", unilamellar vesicles were prepared for the interaction studies by thin film hydration. Z-average size and zeta potential (see Table 1) of the liposomes were determined directly after homogenization of the liposomes by extrusion in the respective buffers. Z-average of all liposomes was ≤130 nm and PDI ≤ 0.15.

3.4.1. Microscale thermophoresis

Microscale thermophoresis allows the quantitative investigation of interactions of proteins or peptides in free solution with other molecules e.g. phospholipids at low sample volumes [27,28]. Label-free measurements have the additional advantages that no sample preparation is required, that the site of interaction on a molecule is not disturbed or hindered by the attachment of a fluorescent label to the protein or peptide and that mobility is not restricted [28]. Native binding behavior is measured with label-free MST. It has been demonstrated before that even liposomes with a size of several 100 nm can be analyzed with MST [29].

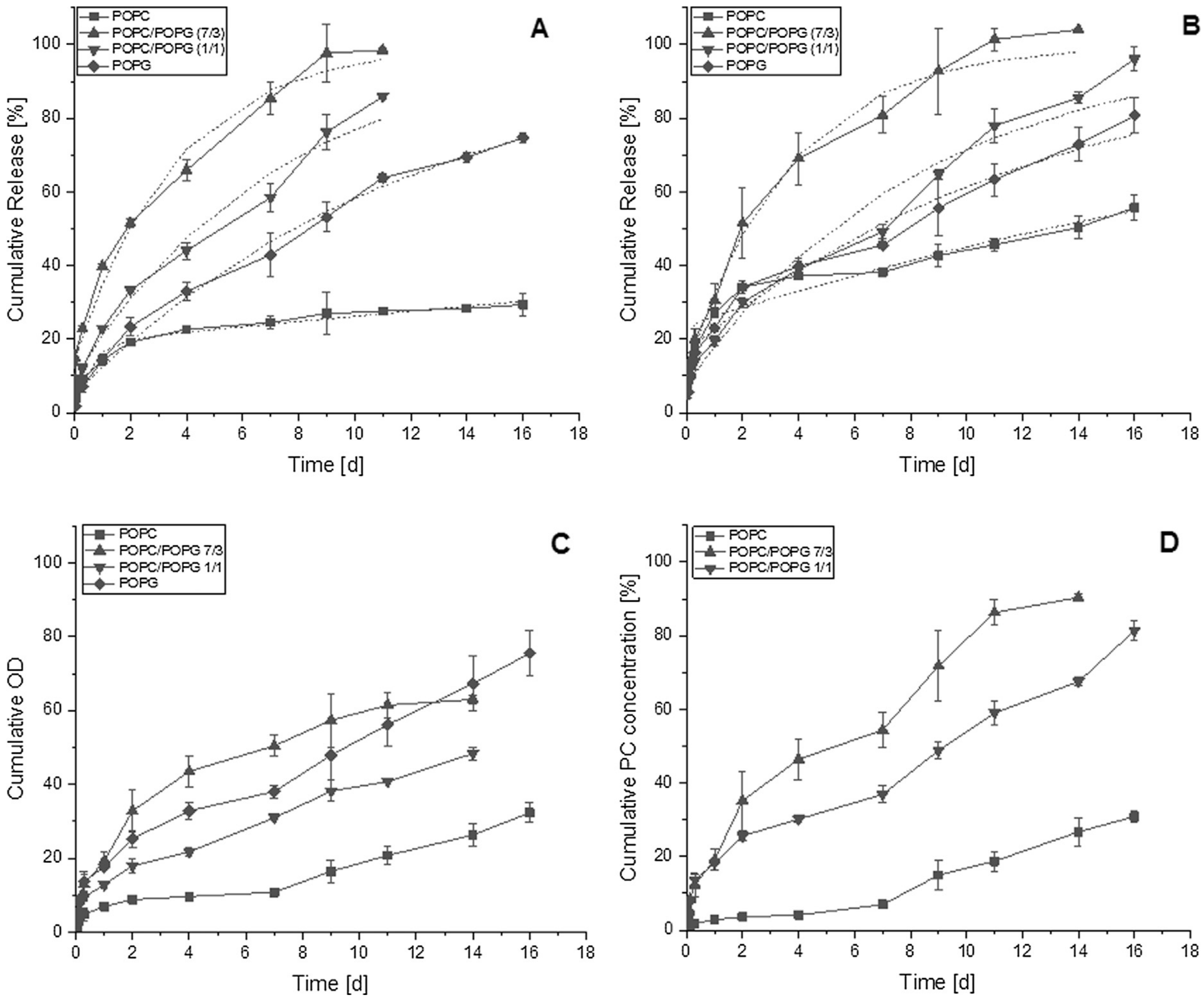

Fig. 4. In vitro release of exenatide from VPG and erosion behavior of VPG during release. (A) Cumulative exenatide release from VPG. Exenatide was encapsulated at pH 4.5 in all formulations. (Release medium: 20 mM PBS pH 7.4). Dashed lines show fit with Eq. (1). (B) Cumulative exenatide release from VPG. Exenatide was encapsulated at pH 7.4 in all formulations. (Release medium: 20 mM PBS pH 7.4). (C) Cumulative optical density of the released fractions obtained from in vitro release of exenatide. (D) Cumulative PC concentration obtained from phospholipid assay for all formulations containing POPC is shown in percentage of total PC in the VPG formulation. (Mean ± SD; n = 3).

Table 1
Zeta potential of the liposomes used for interaction studies. (Mean ± SD, n = 3).

Liposome composition	Zeta potential [mV]	
	pH 4.5	pH 7.4
POPC	−0.6 ± 0.6	−5.6 ± 2.2
POPC/POPG 7/3	−6.2 ± 1.2	−17.6 ± 0.8
POPC/POPG 1/1	−6.5 ± 1.7	−20.5 ± 0.8
POPG	−7.2 ± 1.7	−25.4 ± 1.7

Since label-free MST is not possible if both binding partners show similar tryptophan fluorescence or if background fluorescence by the buffer components leads to increased noise, control experiments have to be performed before measuring. Further, optimal buffer conditions have to be determined before the experiments [30]. To prevent unspecific adsorption to the capillary walls, Pluronic F127 was added to the buffer before measurement. At low liposome concentrations, the unbound exenatide displays a migration towards higher temperatures, corresponding to a negative thermophoretic signal. Upon binding of exenatide to the liposomes, increasing positive thermophoresis was observed (see Fig. 5). Similar behavior has been reported before for RNA-ligand interactions [31].

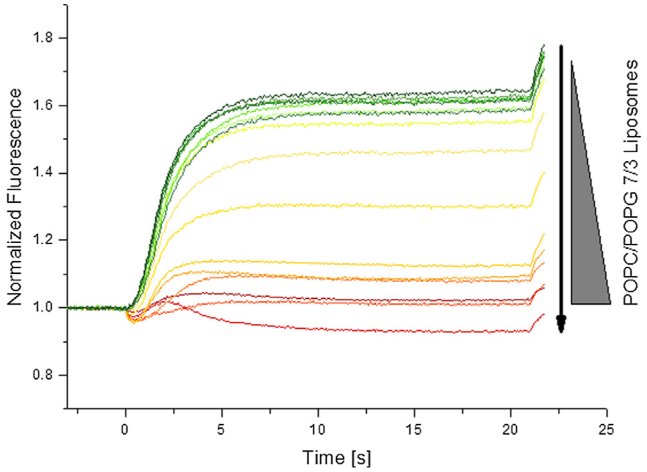

Fig. 5. Raw MST-traces of the titration of exenatide (5 μM) with liposomes composed of a mixture of POPC and POPG 7/3 at pH 4.5. Liposome concentration increases from dark green (30.5 nM) over yellow (7.8–31.25 μM) to dark red (1.0 mM) as arrow indicates. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

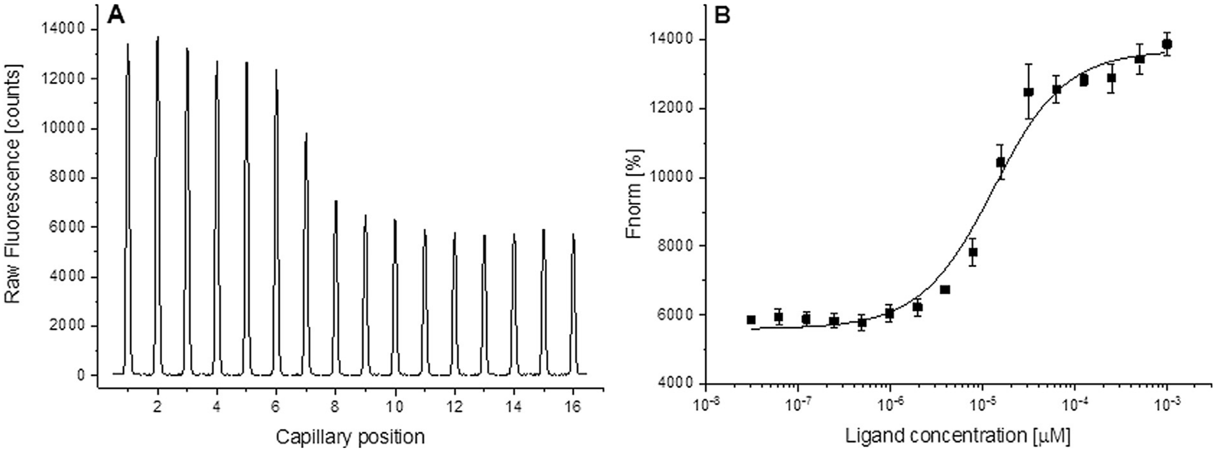

Fig. 6. (A) Raw fluorescence spectra: Initial Fluorescence change with increasing amount of POPC/POPG 1/1 liposomes in exenatide solution (pH 4.5). Liposome concentration decreases from capillary position 1 (0.5 mM) to capillary position 16 (15.3 µM). (B) Fitting function of the initial fluorescence signal of POPC/POPG 1/1 liposomes to exenatide at pH 4.5. (Mean ± K_d Confidence, n = 3).

Different parts of an MST signal can be analyzed [29]. Before the sample is heated, initial intrinsic fluorescence is determined. Usually, the initial fluorescence should be constant for all samples; changes in initial fluorescence occur only if the ligand binds closely to the fluorophore. Artifacts or adsorption of the peptide to the reaction tubes have to be ruled out to account for this phenomenon. In the present study, initial fluorescence changes occurred, indicating a screening of the tryptophan residue in exenatide from water upon binding to liposomes. In this case, the binding constant K_d may already be calculated from the initial fluorescence changes. Raw fluorescence scans of each capillary for the titration of exenatide with POPC/POPG 1/1 liposomes are illustrated in Fig. 6A. These initial fluorescence changes were not observed for any of the formulations at pH 7.4 or for the pure POPC liposomes at pH 4.5. For POPC/POPG mixtures and pure POPG liposomes, binding constants could be derived from a fit of the normalized, initial fluorescence data to the law of mass action. An exemplary fit for the titration of exenatide with liposomes composed of POPC/POPG 1/1 is shown in Fig. 6B.

Primarily, MST-signals describe the fluorescence change at a given position in the sample as a function of time after heating by an IR-laser [27,29]. The following thermophoretic movement lasts several seconds until the laser is turned off again. Changes in thermophoresis indicate a change in size, charge and the hydration shell of a molecule and are therefore highly specific for binding events.

In our case, initial fluorescence and the MST-signal by 5 s was used for data evaluation. K_d-values were determined by a fitting function using the law of mass action [28].

$$\mathbf{K_d} = \frac{\mathbf{c_L c_P}}{\mathbf{c_{LP}}} = \frac{(\mathbf{c_{L0}} - \mathbf{c_{LP}})(\mathbf{c_{P0}} - \mathbf{c_{LP}})}{\mathbf{c_{LP}}} \tag{3}$$

A change of thermophoretic movement was observed for liposomes containing POPG at pH 4.5 (see Fig. 7A), but neither for liposomes of pure POPC nor for formulations at pH 7.4 (see Fig. 7B).

The dissociation constants K_d for all liposomal formulations as obtained by MST are summarized in Table 2. The fits, derived from the software intern fit-function, resulted in binding constants of $K_d \approx 10–30\ \mu M$ for the initial fluorescence analysis and analysis of the MST-signal. We may conclude that all POPG containing formulations investigated here show a high affinity binding of exenatide, while no such binding was detectable for pure, zwitterionic POPC membranes. Significance and meaning of the apparent differences between the dissociation constants are not important for the current study and would need considerable, additional efforts and methods to be elucidated. In particular, the apparently higher K_d for POPG as obtained repeatedly from the MST signal but not from initial tryptophan fluorescence should not be claimed significant without further validation. Potential sources of enhanced error or biases might be an oversimplification of the binding model, incomplete equilibration, aggregation phenomena, etc.

4. Discussion

4.1. *In vitro release behavior of exenatide from VPG*

As discussed above, several previous studies suggested faster, erosion controlled release from low-viscosity gels and slower, diffusion controlled release from more viscous ones [3,32]. Exenatide release from the formulations studied here showed the opposite behavior. The formulations composed of POPC showed significantly lower viscosity than those containing POPG but, nevertheless, substantially slower release than those with POPG. Obviously, viscosity alone is not always sufficient to understand or predict release characteristics. Instead, a complex combination of release mechanisms with their individual kinetics has to be considered for a more detailed understanding.

There are two principal mechanisms of drug release from VPGs, diffusion of the drug out of the gel and erosion of the gel releasing the embedded drug. According to the Einstein-Stokes equation, a peptide of 5 kDa should have a diffusion coefficient in water of the order of $D = 130\ \mu m^2/s$. Taking into account that the mean square distance of diffusion, MSD = 2Dt, this D implies that within a time of t = 1 h, the peptide spreads out over a root mean square distance of RMSD ≈ 1 mm. Depending on the dimensions, tortuosity, and microviscosity of the gel, this should be the order of magnitude of releasing non-entrapped drug from the gel. Our experimental setup was designed to record long-term release and not suited to precisely quantify release on this time scale, but practically all release curves showed a release of the order of 10–20% of the drug with a half-life of up to 1 h.

We found a substantial retardation of release for the majority of the drug in all formulations tested here, by about 2.5 days for POPG containing gels and by several weeks by purely zwitterionic gels of POPC. In the following, we will attempt to interpret this behavior on the basis of additional data.

4.2. *Interactions between exenatide and phospholipids*

In order to assess the contributions of exenatide binding to the lipid gel to the retardation of drug release, we have studied exenatide-liposome interactions in dilute systems by microscale thermophoresis. The binding of exenatide to phospholipid membranes strongly depends on the charge of the peptide and phospholipid. Given that strong binding

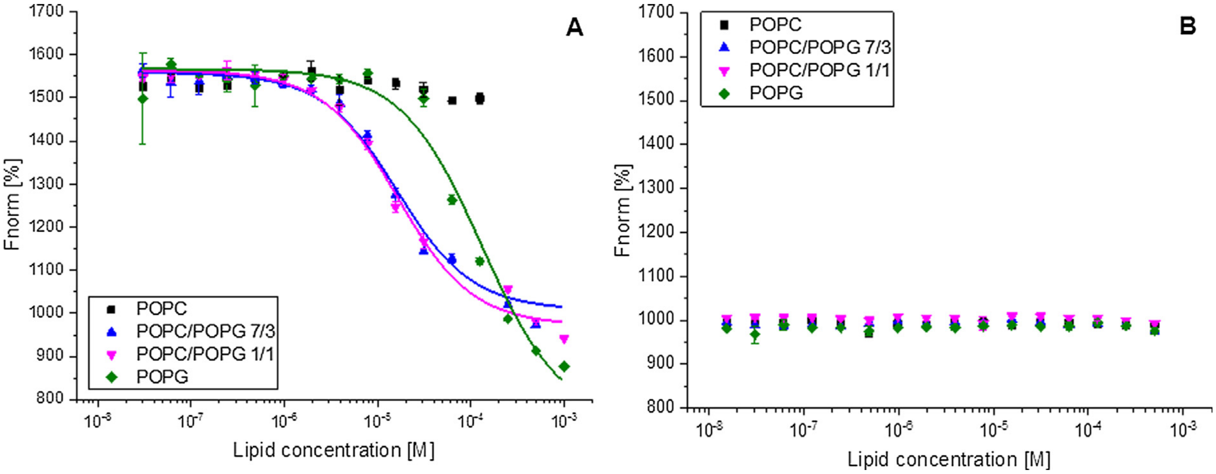

Fig. 7. Comparison of Dose Response determined by MST-Signals of the titration of exenatide (5 µM) with liposomes at (A) pH 4.5 and (B) pH 7.4 (n = 3). Fit functions for the two POPC/POPG mixtures and the pure POPG liposomes are shown for pH 4.5.

Table 2
Summary of K_d-values [µM] derived from the MST measurements. All experiments were performed at pH 4.5 in 50 mM sodium acetate buffer.

Lipid composition	Initial fluorescence K_d [µM]	MST-signal K_d [µM]
POPC	–	–
POPC/POPG 7/3	27.3 (± 3.2)	13.8 (± 2.5)
POPC/POPG 1/1	10.4 (± 2.0)	13.9 (± 2.0)
POPG	13.7 (± 4.0)	128.4 (± 35.2)

was observed only for cationic peptide (pH 4.5) to anionic, POPG containing, membranes supports the idea of a largely electrostatic, superficial adsorption with little or no intrusion of hydrophobic side chains into the membrane.

Binding was detected based on changes in tryptophan fluorescence and thermophoretic mobility with MST measurements. Except for one apparent outlier of unclear significance as discussed above, dissociation constants for all liposomes containing POPG are, within error, of the order 10–30 µM. A detailed and rigorous discussion of the subtle differences between the K_d-values is, however, not warranted and outside the scope of this study.

Binding to zwitterionic POPC is significantly weaker and was not measureable in the investigated concentration range. Such a high K_d may still suffice for binding all peptide in a lipid gel with its far higher concentration but the weaker binding energy would still allow for a faster off rate and, hence, release kinetics from the surface to the bulk solution.

4.3. Erosion-driven, fast release from POPG containing gels

Let us recapitulate the insights obtained for exenatide in POPG containing gels. The retarded release of ≥80% of the drug showed half-lifes of 2.5–8 days and showed a good, linear correlation with lipid erosion into the supernatant ($r > 0.98$, not shown explicitly). At pH 4.5, exenatide binds to POPG-containing liposomes with a high affinity ($K_d \approx$ 10–30 µM). As one should expect, binding to the anionic membrane was not detectable at pH 7.4, where the peptide is negatively charged, too. Nevertheless, release kinetics were virtually unaffected by the initial pH within the gel.

Taken all these points together, we can conclude that the kinetics of exenatide release from POPG containing gels studied here is governed by the kinetics of gel erosion. However, it has to be taken into account that exenatide released in vesicles may contribute significantly to in vitro release data, but not necessarily be fully bioavailable in vivo.

Note that in contrast to zwitterionic lipids swelling spontaneously, only up to a certain level of hydration, anionic lipids may swell infinitely due to their electrostatic repulsion [33]. An excess of bound peptide at pH 4.5 would overcompensate the charge of the lipid and render the overall charge of the peptide-lipid-particles or layers positive. Again, this might promote erosion of the gel. Interestingly, both the strongly bound exenatide in the pH 4.5 VPGs as well as the non-membrane-interacting exenatide in the pH 7.4 VPGs show no substantial diffusional release that would detectably add to that by erosion. Of course, a change in diffusion due to binding will not matter to overall release kinetics as long as erosion remains rate limiting. Second, the pH in the VPGs (4.5 or 7.4) approaches the pH of the surrounding buffer (20 mM PBS pH 7.4) during the first days of release by diffusion. Alignment of the pH of the buffer inside of the VPG to the pH of the surrounding release buffer may explain the strong similarities between the release curves at encapsulation pH 4.5 and 7.4. Third, as discussed in the next section, both very strong binding and repulsion will prevent diffusional release from a multilamellar gel, either by attraction or by barrier function, respectively.

4.4. Barrier based retention in gels of POPC and E80

Gels of the zwitterionic lipid POPC retarded the release of ≈80% of the loaded exenatide to rates that suggest half-lifes of several weeks and the correlation with lipid erosion was less compelling. An affinity of the peptide to these membranes could not be observed at least in dilute liposomal systems implying that interactions in the gel are repulsive or only weakly attractive.

No matter what finally controls release, both erosion and diffusion of these gels are retarded to half-lifes of at least weeks. The lack of high affinity binding of exenatide to these VPGs suggests that it is primarily the barrier function of membranes in the gel that allows for this very slow diffusional release. Note that at least a low affinity of the drug to the phospholipid is vital for the molecule to penetrate into and across the phospholipid bilayer. The partitioning-diffusion model defines membrane permeability P on the basis of partition (here: K) and diffusion coefficient (D):

$$\boldsymbol{P} = \frac{\boldsymbol{K \cdot D}}{\boldsymbol{d}} \tag{4}$$

for a given membrane thickness of d. That means, membrane binding/partitioning is a prerequisite for permeation. For sink conditions, the resulting time dependence of the retained concentration becomes:

$$\boldsymbol{c_{donor}(t) = c_0 \cdot exp\left\{-\frac{P \cdot A}{V_{donor}} \cdot t\right\}} \tag{5}$$

with the initial drug concentration on the donor side of c_0, the membrane area A, and the volume of the donor compartment of V_{donor}. That means, the rate of drug release across a membrane is expected directly proportional to P and hence, to K. The exenatide-liposome system might not obey the partitioning-diffusion model to a good approximation, for example because the peptide might adsorb on the surface rather than partitioning into the membrane. Nevertheless, a higher concentration at the membrane should facilitate permeation compared to the case of a membrane that is not approached by the peptide. In this context, it is also instructive to recall Lipinski's rules that, in the end of the day, describe prerequisites for passive absorption of a drug across a membrane. For fast permeation, it needs intermediate polarity and hydrophobicity values. Too hydrophobic molecules ($\log P > 5$) are little soluble and get stuck in the membrane; too polar molecules (too many H-bond donors and acceptors) prevent the drug from entering the membrane in the first place. Hence, it is very plausible that barrier-controlled release from a lipid membrane based drug delivery system does not increase or decrease monotonically with a certain parameter, such as hydrophobicity.

5. Conclusions

Release from VPGs is an extremely complex process, resulting of different mechanisms like erosion, interactions, diffusion and permeability of the lipid bilayers, all occurring at the same time and complementing each other. Additional factors influencing drug release may be the inner structure e.g. vesicle size and homogeneity as well as the viscosity of the VPG.

A high viscosity of the VPG was previously connected to slow release and erosion rates of the system. This correlation was not confirmed in the present study. For POPG containing VPGs, the release of exenatide showed half-lifes of several days that were, obviously, governed by the erosion of the gel. In spite of the fact that these lipid mixtures bound exenatide with high affinity ($K_d \approx 10–30\ \mu M$) at pH 4.5 but not detectably at pH 7.4, gels produced at these two pH values shared similar release rates. This can be explained by diffusion not being rate limiting and relatively fast pH matching between VPG and release medium due to diffusion of protons or hydroxide.

VPG of POPC showed much slower release of exenatide, with a half-life of several weeks. The fact that this lipid did not show high affinity binding of exenatide suggests that their very strong retardation of the diffusional release of exenatide is primarily based on its very weak permeability across the lipid membranes in the gel.

Notably, both very high and lacking affinity of the drug to the gel retard diffusion, by attraction or barrier function, respectively. Translation of our findings to other proteins or peptides is in general possible within limitations. The two major factors, erosion of the VPG and membrane permeability, contributing to the release from the depot system are independent of the encapsulated drug itself. However, a minor contribution of interactions, molecular weight and other factors to the release kinetics cannot be completely excluded and thus further case to case studies may be needed to strengthen our assumption.

Thus, a mathematical description of the release from VPGs resulting from this complex mixture of mechanisms, contributing to or influencing the release behavior is not possible at the moment. A rational design of the depot formulation based on mathematical predictions of the release behavior for novel drug candidates is still impossible.

Further investigations on the release behavior and characterization of the fluidity and barrier functionality of the multiple lipid bilayers of the VPG are necessary to resolve all mechanisms behind the release kinetics completely, to be finally able to describe release behavior from VPG mathematically.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.06.007.

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