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Quantifying the heterogeneity of enzymatic dePEGylation of liposomal nanocarrier systems

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Abstract

Lipid nanoparticles have been clinically successful in particular recently within the vaccine field, but better tools are needed to analyze heterogeneities at the single particle level to progress drug delivery designs to the next level. Especially, liposomal nanocarriers are becoming increasingly complex e.g. by employing environmental cues for shedding their protective PEG layer, however a detailed mechanistic understanding of how the dePEGylation

varies from liposome-to-liposome is still missing. Here we present the development of a fluorescence microscopy based assay capable of detecting the enzyme mediated dePEGylation of individual liposomes. We employ this methodology to understand how enzyme type-, concentration- and incubation time, in addition to liposome size, affects the dePEGylation at the single particle level.

Keywords

Liposomal nanocarriers

Enzyme mediated dePEGylation

Single liposome measurements

Quantitative fluorescence microscopy

Liposome heterogeneity

Introduction

Bestowing liposomal nanocarriers (LNs) with the ability to avoid being readily removed by the body's natural clearing machinery, hereby prolonging their circulation time, has been paramount for translating LNs to the clinic [1, 2]. Typically, this so called stealth effect is achieved by covering LNs with chemically inert structures, most often the polymer polyethylene glycol (PEG) [2]. However, it has long been recognized that while PEGylation is beneficial for increasing circulation time, it can also greatly reduce LN efficiency by limiting e.g. cellular uptake and endosomal escape [3, 4]. To overcome this 'PEG dilemma' there has been a growing interest in developing smart LNs with cleavable PEG coatings, allowing for selective dePEGylation at the precise LN site of action. A number of environmental cues have been employed as endogenous triggers of LN dePEGylation, including pH, redox potential and

endogenous enzymes [5-8]. However, despite the immense amount of effort invested in these smart LNs we are still not experiencing the projected surge in the number of LN-based nanomedicines being available to patients in the clinic [8, 9].

The translational gap experienced for LN-based nanomedicines has been proposed to be, at least in part, related to a lack of precision and sensitivity in the tools employed to characterize the physicochemical properties [9, 10]. Due to technical limitations, most physicochemical characterization of liposome properties in general, and dePEGylation in particular, have traditionally been done using bulk assays [10]. Such assays are limited to providing an ensemble average readout of how e.g. size and surface charge are modified as PEG moieties are removed from the liposome surface [11]. The validity of these readouts rests on the assumption that dePEGylation is uniform for all liposomes in the sample. Assuming particle uniformity has, however, been challenged in recent years, with reports showing that liposomes in the same batch can display considerable inhomogeneities in many of their physicochemical properties [12-15]. The importance of how intra-sample inhomogeneities affect liposome function is increasingly recognized [10, 16, 17]. In the case of variations in LN dePEGylation having an elevated PEG density could potentially lead to a liposome fraction not capable of releasing its cargo or being taken up by the target cells. On the contrary a diminished PEG density or to rapid a dePEGylation for another liposome fraction will result in a limited therapeutic effect as they will be rapidly cleared due to the absence of sufficient PEG shielding. Overall such inhomogeneities will produce less controlled therapeutic efficiencies, however we are still lacking the experimental setups and thus information on how dePEGylation differs between individual LNs.

Here, we present a novel single liposome assay that can determine the degree of dePEGylation for individual LNs. As a model system, we employ our recently described advanced liposomal LN system for immunotherapy, capable of inducing tumor repolarization showing a strong

therapeutic efficacy in various cancer models [18]. The liposomes were covered with PEG through post insertion of a PEGylated, cleavable lipopeptide (PCL) comprised of a cholesterol insertion motif, a cleavable peptide linker region and a PEG2000 domain. The PCL motif is cleaved by intratumoral proteases, where especially the matrix metalloproteinases (MMPs) are known to be up-regulated in the tumor microenvironment [19], hereby shedding the outer PEG coating and facilitating site specific cell uptake. Here we used the single liposome assay to study dePEGylation of individual liposomes in a massive parallel manner. This allowed us, for the first time, to directly show that two classes of proteases, thermolysin, and MMPs, both can induce an overall monodisperse dePEGylation of a generic LN system, although with very different kinetics. The access to individual liposome properties meant we could determine a non-uniform time dependent dePEGylation between liposomes of different sizes, with thermolysin, but not MMP, showing increased activity on larger versus smaller liposomes. We believe the assay presented here represents a valuable tool for characterizing LNs at a previously unseen level of detail. This could help ensure that future LN are selected to display a high degree of uniformity, helping to produce more precise and controllable particle uptake and drug release at the site of action.

Materials & Methods

Materials

1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dioleoyl-3-trimethylammonium-propane (methyl sulphate salt) (DOTAP), Cholesterol (Chol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000-Biotin), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)2000-N'-

carboxyfluorescein] (ammonium salt) (DSPE-PEG2000-CF) were all acquired from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA).

1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-Atto655 (DOPE-Atto655) and Atto488 NHS-ester were acquired from Atto-Tec (Siegen, Germany).

4-Aminophenylmercuric acetate (AMPA), Bovine Serum Albumin (BSA) (A9418), BSA-biotin (A8549) and streptavidin (S4762) was acquired from Sigma Aldrich (Brøndby, Denmark)

9-Fluorenylmethoxycarbonyl (Fmoc) amino acids and *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were purchased from GL Biochem (Shanghai, China) or Bachem AG (Bubendorf, Switzerland). TentaGel PAP_{2000} resin was purchased from Rapp Polymere GmbH (Tuebingen, Germany). Fmoc-NH-PEG2000-NH_2 was purchased from JenKem Technology USA (Plano, TX).

For information on the synthesis, purification and characterization of PCL) and the Atto488 labeled analog, PCL-Atto488, please see the supplementary information (Fig. S1b).

Liposome formulations

POPC membrane system (unsaturated):

POPC:Chol:DOTAP:DOPE-Atto655:DSPE-PEG2000-Biotin (59.95:32:7.5:0.5:0.05)

It has previously been shown that the incorporation of positively charged liposomes will enhance liposome uptake, but reduce circulation kinetics [20]. Thus, in our group we typically prepare formulations with 5 – 7.5 mol% cationic lipids, shielded by a PEG layer which will either slowly dissociate (Chol-PEG2000) or actively be cleaved off (PCL) and thus reveal the positive liposome and mediate cellular uptake [18, 21-24]. Furthermore, we aimed at post inserting 4 mol% PEG moieties as we have performed in-house pharmacokinetic studies

demonstrating that the resulting PEG surface coverage resulted in prolonged circulation kinetics (unpublished data). Also, 4 mol% falls within the typically employed surface densities tested in previous post insertion studies ranging from 3 mol% [25, 26] to 7.5 mol% [27].

Liposome and PEG-lipid preparations

We prepared a liposome formulation containing POPC:Chol:DOTAP:DOPE-Atto655:DSPE-PEG2000-Biotin (59.45:35:5:0.5:0.05 mol%). For liposome formation, lipids in powder forms were dissolved in tert-butanol:Milli-Q water 9:1, mixed to the desired formulation-ratios in glass vials and lyophilized overnight. The dry lipids were re-hydrated in a HEPES-glucose buffer (10 mM HEPES, 5 % glucose buffer, pH 7.4) to a concentration of 1 mM total lipid and put under 65 °C heating for 1 hour. Size of the liposomes was controlled by extruding 21 times through a 100 nm Whatman filter (GE Healthcare) using an Avanti mini-extruder (Avanti Polar Lipids) on a heating block at 65 °C. Liposomes were transferred to glass vials and stored at 4 °C.

The total lipid concentration of the liposome stocks was determined by measuring the phosphorus concentration using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Samples were diluted 200 times in an ICP-MS diluent (2% HCl, 10 ppb Ga) to fall within a set of standard samples from 25-100 ppb phosphorus, and phosphorus content measured on an ICAP-Q from Thermo Fisher Scientific (Waltham, MA, US).

Hydrodynamic diameter and polydispersity (PDI) of the liposomes were measured as the average from 3 runs of 15 cycles by dynamic light scattering (DLS) using a ZetaSizer Nano ZS from Malvern Instruments (Malvern, Worcestershire, UK) (Fig. S1).

To perform the post insertion of PCL, we prepared a 1 nmol (1:4) mixture of PCL:PCL-Atto488 by dissolving it in tert-butanol:Milli-Q water 9:1 and mixing the desired ratios in glass vials and lyophilizing overnight [28]. Next, based on the lipid concentration from the ICP-MS

measurements, we diluted the liposome samples and added a total volume of the liposome solution that would give a theoretical PEG surface density of 4 mol%, while keeping the concentration of the PEG-lipid around 2 μM and thus below the critical micelle concentrations [25, 27]. Importantly, Atto488 has previously been shown not to interact with membranes [29], thus we anticipated that the attachment of Atto488 would not interfere with the post insertion process.

Collection of human plasma

For control experiment with liposomes incubated in human blood plasma, we followed standard protocols as blood was drawn by certified staff from healthy donors undersigned consent. The identities of the donors were unknown to the researchers performing the experiments, and all requirements for blood collection at the Technical University of Denmark were followed in agreement with the guidelines of National Committee on Health Research Ethics. Blood was collected in Hirudin tubes (Sarstedt, Nürnbrecht, Germany). The blood was transferred to 2 mL Protein LoBind Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 3000 g for 15 minutes in order to separate cells from plasma. The plasma supernatant was transferred to fresh LoBind tubes and stored at 4 °C. Experiments with human plasma were always carried out on the same day as the blood was drawn.

Enzymatic cleavage assays

The PCL construct has a protease cleavage site that allows for liposome dePEGylation to be performed *in vitro* and *in vivo* using different proteases (Fig. S1B). For the *in vivo* administration of PCL liposomes, elevated levels of MMPs in the tumor microenvironment [30] are the proposed driver of liposome dePEGylation [18]. However, as recombinant MMP proteins are inherently unstable, most *in vitro* characterization experiments are typically

performed using more stable members of the protease enzyme family, e.g. thermolysin which shares the same cleavage site as MMP9 [18]. In an attempt to describe and mimic both the *in vitro* and *in vivo* scenarios, we here investigated the dePEGylation of individual liposomes using both thermolysin as well as recombinant MMP2 and MMP9.

For the thermolysin cleavage experiments, we mixed 20 μL of a 1 mM lipid liposome solution with 180 μL HEPES-buffered saline [100 mM NaCl, 50 mM HEPES (pH 7.4), 1mM $CaCl_2$ and 2 μM $ZnCl_2$] supplemented with thermolysin (1.6 μg/ml). The liposomes were incubated with thermolysin at 37 °C in an Eppendorf tube to mimic typical *in vitro* cleavage conditions and avoid potential surface binding of cleaved PCL constructs, interfering with the fluorescence readout from the liposomes. At various time points, 5 μL was extracted from the solution and added to 300 μL HEPES-buffered saline in a chamber on an Ibidi μ-slide 8 well glass coverslip (Ibidi, Cat. No. 80827, Martinsreid, Germany) and immediately imaged.

For the MMP cleavage experiments we employed a combination of recombinant MMP2 and MMP9 proteins (MerckMillipore, Cat. No. PF037 and PF038, Darmstadt, Germany). To activate the MMP enzymes we followed a standard protocol and incubated 0.375 μg MMP2 and 0.96 μg MMP9 in 14 μl HEPES-buffer [10 mM HEPES, 139 mM NaCl (pH 7.4)] with 1 μl 50 mM AMPA in 50 mM 0.1 M NaOH for 1 hour at 37 °C. Subsequently, we added 7.5 μl of the activated MMP solution to 200 μL of a 0.01 mM lipid liposome solution, again took out 5 μL at various time points, and added to 300 μL HEPES-buffer in an Ibidi chamber and immediately performed the imaging.

Neither the presence of thermolysin nor MMPs seems to affect the BSA-biotin and streptavidin linkage as the amount of liposomes immobilized on the surface was constant even for prolonged acquisition periods.

Single liposome assay

We used the single liposome assay as previously described [13, 31-35]. In brief, liposomes were labeled by including 0.5 mol% of DOPE-Atto655 and immobilized on a passivated glass surface (Fig. 1a). We extracted the integrated fluorescence intensity of the membrane dye, which scales with the surface area [33]. For each liposome we also extracted the integrated PCL-Atto488 fluorescence intensity, which scales with the number of post inserted PCL-Atto488 motifs. Now taking the ratio between the integrated PCL-Atto488 and DOPE-Atto655 intensities gave us the absolute density in arbitrary units [31, 32, 34, 35].

To determine the size of each individual liposome we also used that the integrated intensity of the DOPE-Atto655 membrane dye scales with the liposome surface area, thus the liposome diameter is equal to the square root of the integrated intensity multiplied by a correlation factor [33, 36]. As previously described in detail [37], we determine the correlation factor by measuring the average size of a monodisperse reference liposome sample extruded through a 50 nm filter using DLS, before imaging the same reference sample using identical microscopy settings as used for the actual sample. The accuracy of this calibration approach has been validated using cryo-electron microscopy and from this independent technique, we estimated a ± 5 nm uncertainty on the liposome diameter determined by the fluorescence approach [33].

Equipment and settings

For imaging liposomes we used a Leica TCS SP5 inverted confocal microscope and an oil immersion objective HCX PL APO CS × 100 (NA 1.4). Detection of Atto488 labels was performed at 495–580 nm (exc. 488 nm); detection of Atto655 labeled vesicles was performed at 640–750 nm (exc. 633 nm) using photo-multipliers. In all cases, sequential imaging was used to avoid cross excitation. Images had a resolution of 2048 × 2048 pixels, with a pixel size

of 25.2 nm and a bit depth of 16. Image analysis and data treatment were performed using Fiji (ImageJ) and custom-made routines in Igor Pro (Wavemetrics). The integrated intensity of individual liposomes was extracted by 2D gaussian fitting of the diffraction limited spots and thresholding, minimum particle area and ellipticity were used to faithfully track and colocalize particles between channels.

Results & Discussion

To quantify the dePEGylation of individual liposomes we employed a previously described fluorescence based single liposome microscopy assay [28, 37, 38]. In brief, we immobilized individual liposomes on a passivated glass surface and imaged them by fluorescence confocal microscopy (Fig. 1). A recently developed liposome drug delivery platform containing post inserted PCLs [18] served as an enzyme labile model LN system, allowing us to quantify how protease type-, concentration- and incubation time, as well as the liposome size, affected dePEGylation. To do this at the single particle level, we needed to determine the PCL density on individual liposomes. Therefore, we substituted a fraction (1:4) of the post inserted PCL constructs with a fluorescently labeled analog, PCL-Atto488, and additionally labeled the liposomes with a reference membrane dye (DOPE-Atto655) (Fig. 1a). Because immobilization does not perturb the spherical shape of the individual liposomes [39], the extracted integrated Atto655 membrane dye intensity can be directly correlated to a relative liposome surface area [31, 33-35]. We measured the total amount of inserted PCL as the integrated Atto488 intensity, thus, by taking the ratio of the Atto488 and Atto655 integrated intensities we could calculate the PCL density for each individual liposome (Fig. 1b) [28]. The high throughput manner of the assay allowed us to image ~10^3 liposomes for each experimental condition. The main experimental limitation of the presented assay is the need for incorporating a fluorescent reporter molecule in the liposome and on the construct of interest. Here it is important to ensure

that the fluorescent probes that are used display as small an influence on the studied system as possible. Thus it is important to choose fluorophores that display minimal interaction with lipid membranes [40], are bright and photostable to avoid issue with photo-toxicity and has low intrinsic fluorophore-fluorophore interaction to avoid demixing and quenching. Here we employed the assay to track, for the first time, if the PEG surface density changed uniformly for all individual liposomes of the ensemble.

The *in vivo* cleavage of the PCL liposomes is believed to be facilitated by the MMPs overexpressed at the tumor site [18, 30]. Recombinant MMPs are however unstable, so most *in vitro* studies of MMP activity employ more stable proteases, like thermolysin, as model systems [18]. Thus, we first investigated how thermolysin cleaved the PCL construct on individual liposomes. Initially, we imaged control liposomes not incubated with thermolysin (0 hours) and extracted the PCL density on each individual liposome, which we plotted as a histogram (Fig. 1b and 2a). We normalized the histogram mean to 1 using the fitted Gaussian function and to ease the comparison between experiments we used this normalization factor for all subsequent histograms for thermolysin. As previously described in detail [28], the post insertion method of PCL leads to a significant degree of inhomogeneity, seen as a spread in the density values around the ensemble mean (Fig. 2a). Next, we matched previously described *in vitro* cleavage conditions [18] and incubated liposomes with 14.4 μg thermolysin per μmol lipid in an Eppendorf tube at 37 °C, taking out liposome samples at different time points. These samples were then added to microscopy surfaces for immobilization and confocal microscopy (Fig. 1b). In the normalized PCL density histograms for 4 or 12 hours of incubation, we detected a clear enzyme mediated reduction in PCL density as the distributions shifted towards lower values (Fig. 2a and Supplementary Information Table 1). Non-enzyme mediated dePEGylation did not contribute to the reduction in PCL density over time, as a control experiment incubating liposomes in buffer without enzymes for 24 hours revealed no

significant change in PCL densities (Fig. S2). Interestingly, the PCL densities quantified for liposomes incubated with thermolysin displayed only a single distribution, well fitted with a single Gaussian function (Fig. 2a). This lack of multi-peak distributions suggests that the thermolysin mediated dePEGylation is overall uniform at the single liposome level and is not only occurring e.g. on a selected fraction of liposomes. Such non-uniform dePEGylation could be detected for another known dePEGylation strategy, where a non-cleavable PEG construct slowly leaked out of the liposome membrane when incubated in blood plasma, leading to PEG surface density histogram with multiple peaks (Fig. S3). These data demonstrate the ability of the presented single liposome assay to provide unique insights on the liposome-to-liposome variation for different dePEGylation pathways and it confirms that enzyme mediated dePEGylation of PCL represents a well-controlled mechanism, producing a monodisperse population of dePEGylated liposomes.

We quantified the incubation-time dependent reduction in PCL density by extracting the mean PCL density value from the Gaussian fit to the individual histograms acquired for different incubation time points (Fig. 2a and Supplementary Information Table 1). We then normalized these mean PCL density values to the one found at 0 hours, allowing us to plot the average fraction of remaining PCL as a function of time (Fig. 2b). This revealed an efficient and systematic dePEGylation by thermolysin over time, as the average fraction of remaining PCL density at 12 hours was reduced to 0.11 ± 0.01. After 12 hours, the dePEGylation reached a plateau where only a minor additional reduction in PCL density could be detected (24 hours = 0.08 ± 0.01), suggesting that thermolysin could not induce complete dePEGylation at these conditions. The remaining PCL-Atto488 density is not an imaging artifact as a control experiment with liposomes containing only DOPE-Atto655 displayed no cross-talk signal above the background in the PCL-Atto488 channel (Fig. S4). To extract the dePEGylation kinetics we fitted figure 2b with an exponential function and extracted $T_{1/2}$ = 3.45 hours,

quantifying that the PCL density is halved every 204 minutes. This demonstrates the efficient dePEGylation achieved with thermolysin and the accuracy by which it can be tracked using the single liposome assay.

Despite the lack of multiple distributions in the PCL density histograms, the enzymatic dePEGylation could still lead to increased liposome-to-liposome PEG density variations. To evaluate if this was the case, we calculated the degree of PCL density inhomogeneity (DI) using the coefficient of variation, a statistical term that relates the width (SD) and the mean of the Gaussian function fit to the PCL surface density histograms (DI = SD/mean) [28, 38]. A higher DI denotes a larger variation in the PCL surface densities between individual liposomes and thus a less uniform PCL surface density. However, it is important to note that the DI value is also affected by the error on the determination of the integrated intensities for the individual liposomes, as lower intensities will result in a proportional larger error. Thus, the DI will intrinsically increase as the recorded intensities decreases and to correct for this we prepared a control experiment where different amounts of PCL were post inserted into liposomes and imaged without enzyme incubation (Supplementary Table 2). In figure 2c we plotted the experimental and control DI values versus the remaining fraction of PCL (for the control, this represents the fraction post inserted as compared to the highest post inserted amount). We saw a similar increase in both the experimental and control DI values as the fraction of PCL was reduced, suggesting that the larger liposome-to-liposome variation quantified at lower fractions of remaining PCL correlates with the increased error on the lower intensity signals. Therefore, we conclude that thermolysin mediated cleavage of PCL leads to little liposome-to-liposome variation in PEG density and that thermolysin represents an efficient and reliable dePEGylation reagent to be used in *in vitro* experiments.

Next, we investigated whether increasing the thermolysin concentration affected PCL cleavage efficiency, kinetics or could induce a non-uniform dePEGylation between individual

liposomes. We again performed a time dependent experiment, but now incubated the liposomes with a 1000x fold higher thermolysin concentration (14.4 mg thermolysin per μmol lipid). We plotted the PCL density histograms at various time points and saw a single distribution of the remaining PCL density values, which rapidly decreased, demonstrating the extremely efficient dePEGylation at this thermolysin concentration (Fig. 3a). The data revealed that after only 4 hours of thermolysin incubation we reached an average fraction of remaining PCL of 0.08 ± 0.03 (Fig. 3b and Supplementary Information Table 1). Interestingly, even this very high enzyme concentration and the highly efficient dePEGylation it induces, was not enough to completely remove PCL, as we reached a steady state after 4 hours of incubation, suggesting this to be the lowest obtainable dePEGylation level (Fig. 3b). Fitting an exponential function to the time dependent average remaining PCL densities we quantified $T_{1/2}$ = 0.81 hours, quantifying that the remaining PCL density was halved every 49th minute, thus the 1000x higher thermolysin concentration lead to a 4.2 fold increase in the dePEGylation kinetics (Fig. 3b).

To investigate how PCL was cleaved on individual liposomes by its designed *in vivo* enzyme target, we employed a mixture of recombinant MMP2 and MMP9. We activated the MMPs using AMPA following standard protocols, before incubating the enzyme solution with liposomes. Again, we followed the time dependent dePEGylation, plotting PCL density histograms for various incubation time points (Fig. 4a). Even though we employed a 25 fold higher total MMP concentration as compared to the 1x thermolysin scenario (357.5 μg MMP versus 14.4 μg thermolysin per μmol lipid) we detected a much smaller reduction in PCL density values for the MMP versus thermolysin mediated dePEGylation. This is exemplified by the average fraction of remaining PCL after 24 hours of incubation being 0.57 ± 0.02, demonstrating that even after 24 hours of incubation more than half the PCL constructs could still be detected intact on the liposome surface. The exponential fit to the average remaining

PCL density values for the various time points revealed slow dePEGylation kinetics with $T_{1/2}$ = 13.5 hours, demonstrating that the MMPs halved the PCL density every 810 minutes (Fig. 4b). It is indeed promising that recombinant MMPs can induce dePEGylation and we do note that the *in vitro* measurements are potentially underestimating the *in vivo* level of dePEGylation. This is due to the instability of the recombinant MMPs, which cannot be replenished with fresh enzymes as in the *in vivo* scenario. Another parameter that could affect the accuracy of the *in vitro* MMP dePEGylation quantification is the correlation between the *in vitro* and *in vivo* enzyme:liposome ratio. We used literature- and previous experimental values for the PCL liposomal system to estimate that the employed MMP:lipid ratio in the single liposomes assay was within an order of magnitude of the ratio found in an average *in vivo* tumor (see calculation in SI). Despite the assumptions underpinning this estimation, it does illustrate how it is possible to perform *in vitro* experiments mimicking to some degree, an important aspect of the *in vivo* scenario, e.g. the MMP concentration. Here we show how we can combine access to such *in vivo*-like scenarios with the unique ability of single particle *in vitro* assays to study specific parameters, like dePEGylation, on a level of detail not accessible *in vivo*.

Despite the relatively inefficient MMP mediated dePEGylation we still saw a monodisperse dePEGylation as only a single peak histogram was detected for all time points. This overall homogenous dePEGylation was further confirmed by comparing the quantified DI values from the MMP mediated cleavage with the previously described control experiment (Fig. 4c). For the range of average remaining fraction of PCL density values displayed by the MMP experiment, we saw an overall constant DI value, which matched the control experiment. Thus, we conclude that also the *in vivo* enzyme target for liposome dePEGylation induces a low liposome-to-liposome variation in PCL density. This suggests that enzyme mediated liposome dePEGylation heterogeneity at the tumor site would be minimal, which would help to ensure a

more controlled and uniform liposome:cell interaction and drug release between liposomes of the ensemble.

The single particle nature of the assay not only offered unique insights as to how enzyme properties affected dePEGylation, but also allowed us to study how specific liposome properties affected the process. One liposome property not easily investigated using traditional bulk assays is liposome size [32]. Liposome size, and hence membrane curvature, has been shown to broadly affect enzyme kinetics [41]. However, attempting to study liposome size dependencies by producing liposome populations with different sizes through extrusion through filters with different pore sizes have been shown to bias the outcome of these studies [32]. This is due to large population overlaps, with liposomes extruded using 100 nm and 200 nm filters displaying up to 70 % overlap in their size distribution [31]. Here we took advantage of both the intrinsic size polydispersity of the liposome preparation and the ability of the single liposome assay to assign an accurate size to each individual liposome to study how liposome size affected dePEGylation. To determine the diameter of all individual liposomes in a measurement we first extracted and plotted the DOPE-Atto655 integrated intensities in a histogram, which analogous to previous studies displayed a log-normal distribution (Fig. 5A)[37]. As previously described [33, 37] we next obtained a correlation factor between the integrated DOPE-Atto655 intensity and liposome diameter by employing a reference sample, produced by extruding liposomes 20 times through a 50 nm filter to ensure as narrow a size distribution as possible. The mean diameter of the reference sample was measured using Dynamic Light Scattering (DLS) and correlated to the mean integrated intensity calculated from an intensity histogram obtained by imaging the reference sample at the microscope under identical conditions as the experimental samples [33, 37]. Using the correlation factor we transformed the integrated intensity of each individual liposome to diameter and plotted these in a histogram, revealing a typical size range between ~50 - 350 nm (Fig. 5B). To be able to

determine how the enzyme mediated dePEGylation was affected by liposome size we first evaluated the PCL-Atto488 density before incubating liposomes with either thermolysin or MMP. To ease the quantitative comparison between measurements and time points we divided each data set into five size bins and calculated the average PCL-Atto488 density for each bin. We normalized these average PCL-Atto488 densities by the value found for the largest liposome bin, before plotting the values against the average liposome bin size (Fig. 5C). This revealed an increase in PCL-Atto488 density as the liposome size decreased, quantified as a 1.92 ± 0.28 fold higher density for the liposome bin with an average size of 59.3 nm versus 261.2 nm. A negative control where PCL-Atto488 was premixed in the lipid mixture prior to forming the liposomes revealed no significant liposome size dependent variation PCL-Atto488 density. Our finding that decreasing liposome size leads to increased PCL-Atto488 density when it is post inserted in preformed liposomes corroborates earlier findings for other lipidated peptide constructs [35, 42]. The size dependent distribution of PCL-Atto488, here revealed by the unique capability of the single liposomes represents a source of inhomogeneity in the PEG density between liposomes. While this liposome-to-liposome variation is not enough to introduce a non-monodisperse PEG distribution in our liposomal formulation, it could for other LN systems displaying a more polydisperse size distribution or a distribution skewed towards smaller particles. This again highlights the importance of characterizing LNs at the single particle level.

To study if the liposome size dependency of the PCL-Atto488 density was affected by the enzyme mediated dePEGylation we extracted the fold increase in PCL-Atto488 density between the smallest and largest liposome size bin for each time point of the kinetic measurement (Fig. 5D). The MMP mediated dePEGylation did not affect the size dependent fold increase in PCL-Atto488 density, revealing a near constant value of ~2 over time (Fig. 5D). On the contrary, the thermolysin mediated dePEGylation induced a substantial increase

in the size dependent PCL-Atto488 density, reaching a fold-increase of 4.3 ± 0.9 after 24 hours. We speculate that the different responses observed for thermolysin and MMP to liposome size could be due to thermolysin (~35 kDa) being only half the size of MMP (~77 kDa). Membrane curvature decreases the PEG surface packing due to geometrical constraints [41, 43], which could allow for increased access to enzyme cleavage site below the PEG layer on curved versus flat membranes. Intuitively it makes sense that such an effect would be seen more pronounced for a smaller enzyme, which correlates with our observation that we measure liposome size dependent dePEGylation for the smaller thermolysin enzyme. Overall these data demonstrate, for the first time, how liposome size can strongly influence the enzyme mediated dePEGylation.

To better understand the origin of how thermolysin mediated dePEGylation affected the liposome size dependency we plotted the average absolute PCL-Atto488 density as a function of thermolysin incubation time for both the smallest and the largest liposome size bin (Fig. 5E). As can be seen, the PCL-Atto488 density is reduced on both small and large liposomes systematically, so to better elucidate any differences, we calculated the fraction of remaining PCL-Atto488 density on small and large liposomes by normalizing all values with the respective density found at 0 hours (Fig. 5F). This representation demonstrates a relatively larger decrease in PCL-Atto488 density over time for the largest as compared to the smallest liposomes. Thus the increased size dependent PCL-Atto488 density detected at 24 hours for thermolysin originates primarily from an increased activity on larger liposomes. Such uneven liposome-to-liposome dePEGylation could, in combination with the increased uncertainty for low intense particles, help explain why we see an increased DI over time (DI_{0h} = 0.27 ± 0.02 versus DI_{24h} = 0.40 ± 0.02) (Supplementary Table 2). Overall, these data highlight the importance played by liposome size on both PEG surface density and on the environmental

cue-mediated dePEGylation, further underscoring the need for assays capable of characterizing LNs platforms as the single particle level.

Conclusion

Here we presented a high throughput assay capable of detecting the enzyme mediated dePEGylation of LNs at the single particle level. This allowed us to quantify how uniform the dePEGylation is between individual liposomes of the ensemble. We employed the assay to show how two members of the protease family, thermolysin and MMPs, can cleave the PCL in an overall monodisperse manner. Additionally, we show that liposome size regulates the initial PCL-Atto488 density and can also affect the enzyme mediated dePEGylation. The modular build of the assay allows for it to be easily expanded for studying the dePEGylation of other LN systems as long as these can be anchored on the glass surface and be systematically labelled with a reporter dye. The assay and results presented here serve as important controls for the use of enzyme mediated dePEGylation as a route for producing LNs with a well-controlled and precise function.

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

J.B.L. designed research; J.B.L. performed research; R.E. contributed new reagents/analytic tools; J.B.L., and T.L.A. analyzed data; and J.B.L., R.E. and T.L.A. wrote the paper. All authors discussed the results and commented on the manuscript at all stages.

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Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Captions

Figure 1 | Single liposome assay for detecting the uniformity of the enzyme mediated dePEGylation between individual liposomes (**a**) Scheme depicting the microscopy based assay. Liposomes with post inserted PCL can undergo enzyme-mediated dePEGylation via cleavage of the linker region leading to release of the PEG-chain from the liposome surface. To detect dePEGylation, individual liposomes are immobilized on a glass surface via BSA-biotin:streptavidin linkage. Liposomes are detected using a DOPE-Atto655 membrane dye and a fraction of PCL is labelled with Atto488. (**b**) Micrographs of single liposome depicted for both the DOPE-Atto655 channel (top) and PCL-Atto488 channel (bottom) at different incubation time points, illustrating how enzyme mediated dePEGylation leads to a reduction in PCL-Atto488 intensity over time. Scale bars are 2 μm.

Figure 2 | Thermolysin mediated dePEGylation produces a monodisperse liposome population when studied at the single particle level (**a**) Representative examples of normalized PCL-Atto488 density histograms (red) for incubations with the 1x thermolysin concentration for 0 hours (top), 4 hours (middle) or 12 hours (bottom). All histograms are fitted with Gaussian functions (black) to extract the mean fraction of remaining PCL-Atto488 density. All histograms are normalized to the mean value of the 0 hours histogram. (**b**) The average fraction of remaining PCL-Atto488 density (red circles) plotted against incubation time for all tested incubation periods. The data is fitted with an exponential decay function (black) to quantify the dePEGylation kinetics. (**c**) The average DI values quantified from the Gaussian fits to the normalized PCL-Atto488 density histograms are plotted against the fraction of remaining PCL-Atto488 density for either the thermolysin (red circles) or control (black squares) experiments. All errors displayed represent the standard error of the mean (SEM) for at least three independent experiments, all with over 1000 single liposomes.

Figure 3 | Increasing thermolysin concentration induced faster dePEGylation kinetics for all individual liposomes

(**a**) Representative examples of normalized PCL-Atto488 density histograms (black) for incubations with the 1000x thermolysin concentration for 0 hours (top), 1 hour (middle) or 24 hours (bottom). All histogram is fitted with a Gaussian function (black line) to extract the mean fraction of remaining PCL-Atto488 density. (**b**) The average fraction of remaining PCL-Atto488 density (black circles) plotted against incubation time for all tested incubation periods. The data is fitted with an exponential decay function (black) to quantify the dePEGylation kinetics.

Figure 4 | MMPs facilitate a weak but monodisperse dePEGylation *in vitro* (**a**) Representative examples of normalized PCL-Atto488 density histograms (blue) for incubations

with MMPs for 0 hours (top), 1 hour (middle) or 24 hours (bottom). All histogram is fitted with a Gaussian function (black) to extract the mean fraction of remaining PCL-Atto488 density. (**b**) The average fraction of remaining PCL-Atto488 density (blue circles) plotted against incubation time for all tested incubation periods. The data is fitted with an exponential decay function (black) to quantify the dePEGylation kinetics. (**c**) The average DI values quantified from the Gaussian fits to the normalized PCL-Atto488 density histograms are plotted against the fraction of remaining PCL-Atto488 density for either the MMP (blue circles) or control (black squares) experiments. All errors displayed represent SEM for at least three independent experiments, all with over 1000 single liposomes.

Figure 5 | Liposome size regulates PCL-Atto488 density and can affect the enzyme mediated dePEGylation (**a**) The integrated DOPE-Atto655 intensity extracted for each individual liposome in a representative measurement is depicted as the square root of the intensity in a histogram and fitted with a log-normal function. (**b**) Using the calculated calibration factor all values from (**a**) are translated into liposome size and depicted as a histogram of liposome diameters. (**c**) The average PCL-Atto488 density quantified for five different size bins, here shown normalized to the value found for the largest size bin and plotted against the average liposome diameter for each bin. (**d**) The normalized PCL-Atto488 value for the smallest size bin plotted as a function of enzyme incubation time for thermolysin (red) and MMP (blue). (**e**) Absolute PCL-Atto488 density plotted for the smallest (black) and the largest (green) liposomes of the thermolysin data as a function of incubation time. (**f**) The fraction of remaining PCL-Atto488 calculated as the PCL-Atto488 densities normalized to the value at '0 hours' plotted for the smallest (black) and largest (green) size bin for the thermolysin data. Error bars for all data points in the figure are SEM, however we note that some error bars are on the size of the marker.