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Enzyme-Responsive Hydrogel Microparticles for Pulmonary Drug Delivery

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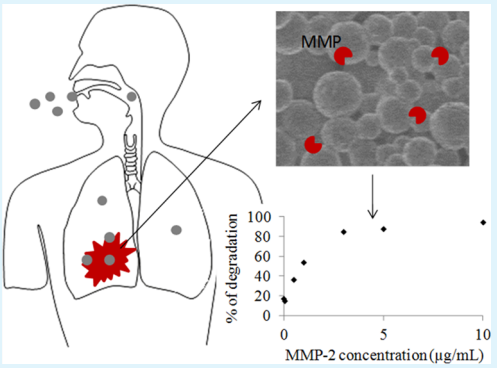
Supporting Information

ABSTRACT: Poly(ethylene glycol) based hydrogel microparticles were developed for pulmonary drug delivery. Hydrogels are particularly attractive for pulmonary delivery because they can be size engineered for delivery into the bronchi, yet also swell upon reaching their destination to avoid uptake and clearance by alveolar macrophages. To develop enzyme-responsive hydrogel microparticles for pulmonary delivery a new synthesis method based on a solution polymerization was developed. This method produces spherical poly(ethylene glycol) (PEG) microparticles from high molecular weight poly(ethylene glycol) diacrylate (PEGDA)-based precursors that incorporate peptides in the polymer chain. Specifically, we have synthesized hydrogel microparticles that degrade in response to matrix metalloproteinases that are overexpressed in pulmonary diseases. Small hydrogel microparticles with sizes suitable for lung delivery by inhalation were obtained from solid precursors when PEGDA was dissolved in water at a high concentration. The average diameter of the particles was between 2.8 and 4 μm, depending on the molecular weight of the precursor polymer used and its concentration in water. The relation between the physical properties of the particles and their enzymatic degradation is also reported, where an increased mesh size corresponds to increased degradation.



KEYWORDS: *poly(ethylene glycol) microparticles, pulmonary delivery, MMP, enzyme-sensitive, emulsion*

1. INTRODUCTION

Nano- or microparticles are widely studied for a variety of medical applications, including imaging,[1] biodetection,[2] tissue engineering,[3,4] and drug delivery.[5,6] Specifically, nano- or microparticles are of interest because they provide a way to overcome some of the major challenges in drug delivery. Currently, pharmaceutical technology relies mainly on the systemic delivery of drug. However, this approach is not capable for providing sufficient quantities of drugs at the desired location in the body for drugs with limited therapeutic windows or where biological barriers must be crossed. To increase the efficacy of pharmaceutical therapies, a major goal is the development of delivery vehicles that can protect a therapeutic from degradation, deliver it to specific tissues, and then release it in a controlled fashion. Further, the use of particles for pulmonary delivery is of particular interest for both local and systemic treatments. In the case of local treatment of diseases such as lung cancer, tuberculosis, asthma, or chronic obstructive pulmonary diseases (COPD), inhaling the drug is advantageous as it allows the drug to go directly to the site of action compared to a systemic administration. Additionally, this localized delivery to the lungs for pulmonary diseases would allow for enhanced bioavailability, decreased side effects, in addition to limiting accumulation in the liver, kidney, or spleen. Pulmonary delivery has also been explored as a method for the systemic delivery of drugs into circulation because of the lung's natural permeability to small molecules, peptides and proteins.[7] Systemic delivery via pulmonary inhalation has other advantages including the fact that lungs offer a highly vascularized large surface area for drug absorption, an epithelial barrier of low thickness, and the absence of the first-pass effect that plagues oral delivery methods.[8,9]

To ensure efficient deposition of a drug carrier into the lungs via inhalation delivery, one of the key parameters for optimization is the carrier's aerodynamic diameter, which is proportional to the particle's diameter and the square root of its gravimetric density. It has been shown that to achieve efficient deposition deep into the bronchi that the optimal aerodynamic diameter of the inhaled particles should fall between 0.5 and 5 μm.[10−13] Particles smaller than 0.5 μm tend to be exhaled, whereas particles larger than 5 μm are unable to reach the smaller bronchioles and alveoli. Aerosolized drugs or polymers such as poly(lactic-*co*-glycolic) acid (PLGA), polyacrylate, or polystyrene and silica have been studied to this end.[14] Once deposited into the lungs, it is necessary that the carrier is able to avoid uptake by alveolar macrophages. Two key factors that can be tailored to avoid macrophage uptake are the size and stiffness of the carrier. It has been shown that stiffer particles, with higher moduli, in the 0.5 to 5 μm range of size, are subjected to a rapid clearance by alveolar macrophages.[15] To

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avoid phagocytosis, particles must also have a geometric diameter larger than 6 μm and present a hydrophilic surface.[16−18] Hydrogel microparticles are therefore attractive candidates for pulmonary delivery, in addition to having low moduli, they can be synthesized with sizes in the range from 0.5 to 5 μm for efficient deposition into the lungs. However, these particles would swell upon deposition into the lungs to reach a size larger than 6 μm, avoiding macrophage uptake. To date, however, only a few studies have been reported on the use of hydrogel microparticles for pulmonary delivery.[13,19−24]

Among the available hydrogels, poly(ethylene glycol) (PEG) was chosen in this study because of its high biocompatibility[25−29] and the fact that it has also been shown to help avoid phagocytosis.[30,31] Here, PEG's ease of modification is also exploited to develop an enzyme responsive drug carrier. For medical applications, stimulus-responsive polymers[32] are promising because they can change their shape or degrade and deliver their payload at a desired site or moment in response to an external stimulus. To this end, pH-responsive,[33] thermally responsive,[33] and enzyme responsive polymers have been studied.[34,35] PEG-based materials can easily be rendered stimuli-responsive by incorporating molecules into the polymer chain. Here, we chose to incorporate a peptide in the polymer backbone, to fabricate an enzyme-responsive drug carrier. In particular, we are interested in obtaining matrix metalloproteinase (MMP)-responsive hydrogel microparticles. MMPs are particularly relevant for pulmonary drug delivery, as they are overexpressed in a large range of pulmonary diseases, including lung cancer, tuberculosis, COPD,[36−39] and for that reason is a target of choice for pulmonary drug delivery. In particular, MMP-2 has been shown to be overexpressed 17-fold compared to healthy tissue in lung carcinoma samples,[40] and MMP-1 and MMP-2 have also been found at high concentrations in the pleural fluids of tuberculosis infected patients.[41]

A variety of methods have already been presented to obtain PEG microparticles with sizes between 1 and 10 μm, including micromachining[42] and solution based approaches.[43−47] Micromachining methods, however, typically require expensive equipment that is not always easy to access. Solution-based methods are therefore advantageous, overcoming the limitations of microfabrication methods. Solution polymerization methods have also been reported as a way to produce PEG microspheres with low molecular weight PEGDA in the liquid form.[48−50] These methods have also been reported for high molecular weight PEGDA precursors, but in these cases, only large microparticles with diameters ranging between 50 and 500 μm were obtained.[51]

Another solution-based technique for the synthesis of polymer microparticles is emulsion polymerization. Emulsion polymerization is particularly attractive due to its ability to produce smaller and less polydisperse particles compared to suspension or precipitation polymerization. Inverse emulsion polymerization has been reported as a way to obtain large PEG microspheres (100−300 μm) by Olabisi et al.[52] In the inverse emulsion polymerization process, the monomer and the photoinitiator are in different liquid phases.[53−55] In the work presented here, a polymerization based on an inverse emulsion is used to make PEG microparticles with sizes below 10 μm from concentrated aqueous solutions of high molecular weight PEGDA macro-monomers. The process is versatile, simple, and easy to implement in any lab with common bench equipment. This process will then be used with PEG chains that incorporate a peptide in their backbone to generate enzymatically degradable microparticles. The peptide is chosen such that it will be cleaved by a collagenase (MMP-1), as the model enzyme. PEG microparticles able to be degraded by small concentrations of MMP-1 have been produced, and the physical properties of those particles can be correlated to their enzyme degradation. We also show that by changing the peptide sequence, the sensitivity of the microparticles to MMP-1 was increased, while they were also degraded by MMP-2.

2. EXPERIMENTAL SECTION

Materials. Poly(ethylene glycol) diacrylate (PEGDA) with a molecular weight of 700, 2000, and 6000 g/mol, silicone oil, hexane, and ethanol were purchased from Sigma-Aldrich. Poly(ethylene glycol) diacrylate with molecular weight of 3400, 5000, and 10000 g/mol, acrylate-poly(ethylene glycol)-succinimidyl valerate (Acr-PEG-SVA) were purchased from Laysan Bio, Inc. Collagenase, 2,2-dimethoxy-2-phenylacetophenone (DMPA), 4-aminophenylmercuric acetate, and silicone oil were purchased from Sigma-Aldrich. MMP-2 was purchased from R&D systems.

Synthesis of Peptide Conjugated Poly(ethylene glycol) Diacrylate. To develop MMP-1 responsive hydrogels a trimer peptide Gly-Leu-Lys (or GLK), containing the enzyme responsive glycine-leucine (GL), was incorporated into the backbone of a PEG-diacrylate macromer. This bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate was formed by allowing Acr-PEG-SVA to react with the Gly-Leu-Lys peptide at a molar ratio of 2:1 in a 50 mM sodium bicarbonate buffer at pH 8 for 4 h under agitation in the dark. Afterward, the solution was dialyzed (molecular weight cutoff = 6−8 kDa) three times against DI water in order to remove any unreacted Acr-PEG-SVA or peptide. After dialysis, the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate was freeze-dried.

In order to increase the sensitivity to MMPs a second peptide sequence with nine amino acids was used: Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys (GPQGIFGQK). The process described above was also used to incorporate this peptide in the backbone of the polymer.

Characterization of the Coupling. The conjugation of the peptide to the PEG chain was confirmed by Fourier Transform Infrared spectroscopy (Nicolet 6700 FT-IR from Thermo Electron), size exclusion chromatography, and via a biuret and a ninhydrin test.

The biuret test is a colorimetric assay used to detect amide bonds in solution. If amides are present in solution, the color will turn from light blue to purple. It was performed as follows: to a solution of the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate in water 3 drops of a NaOH solution (20% in water), and 3 drops of a solution of $CuSO_4$ (1% in water) were added, and the color of the solution was observed. The ninhydrin test is a colorimetric assay to detect amine groups in solution. If amines are present in solution, the yellow color will turn to purple. It was performed as follow: a solution of 10% ninhydrin in ethanol was heated until complete dissolution, this solution was then added to a solution of the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate dissolved in ethanol, and the color of the solution was observed.

The molecular weights of the as-received Acr-PEG-SVA and the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate were determined by size exclusion chromatography (SEC) with multiangle light scattering (MALS). The size exclusion technique, gel permeation chromatography (GPC), was achieved using a Waters 515 HPLC pump connected to Waters Ultrahydrogel 120 and 250 Å columns at 25 °C. An aqueous mobile phase was pumped at a flow rate of 0.5 mL/min. The weight-average molecular weight was determined from multiangle light scattering (MALS) using a Wyatt Technologies miniDawn TREOS light scattering instrument and a Waters 2414 refractive index detector. The polymer samples in aqueous solution were administered by a Waters Rheodyne 7725i manual injector, fractionated by the GPC columns, scattered light from a 658 nm source measured at three angles and the concentration was determined through the refractive index detector. The data was analyzed using Astra software (version 6.2) supplied by Wyatt Technologies.

Microparticle Synthesis. Poly(ethylene glycol) diacrylate (PEGDA) microparticles were obtained by an inverse emulsion method. Briefly, PEGDA was dissolved in water and this solution was then added to silicone oil containing a dissolved photo-cross-linker, DMPA (2,2-Dimethoxy-2-phenylacetophenone). Solutions of PEGDA in water, of different concentrations, were prepared and incubated at 37 °C until complete dissolution of the polymer. At the same time, 50 mg of DMPA was dissolved in 10 mL of silicone oil (with a kinematic viscosity of 1000 cSt at 25 °C). This solution of DMPA in silicone oil was then added to the PEGDA solution, and vortexed for 2 to 20 min at the highest power to form an emulsion. The resultant emulsion was placed under a UV lamp at 365 nm. Finally, the obtained particles were rinsed with hexane, ethanol, and with water. The microparticles were subsequently freeze-dried.

PEG-peptide microparticles were made from the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate in the same way as the PEGDA microparticles before, with a concentration of 70% of bis-poly-(ethylene glycol) acrylate Gly-Leu-Lys in water.

The diameters of the as-formed and freeze-dried microparticles were measured by optical (Nikon Eclipse TE2000) and scanning electron (FEI XL-40 FEG SEM) microscopy, respectively. The size of the particles was determined by analyzing 150 to 250 particles in the micrographs using Adobe Photoshop software. The aerodynamic diameter was calculated from the geometric diameter of the dry particles using the formula: $d_{aer} = d_g\sqrt{(\rho)}$, with d_{aer} the aerodynamic diameter, d_g the geometric diameter and ρ the density of the particles. Here, for the density, we used ρ = 1.12 g/cm^3, the density of poly(ethylene glycol). The diameters of the freeze-dried particles were also analyzed with an aerosizer (TSI PSD 3603 particle size distribution analyzer), which measures the diameter of an aerosolized sample. For each sample, about 30 mg of powder was analyzed.

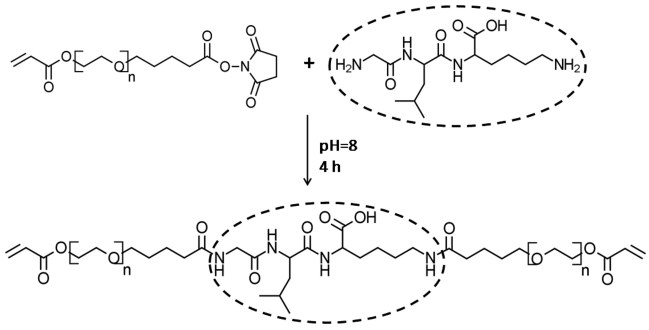
Degradation of the Microparticles in a Collagenase Solution. To study the collagenase degradation, 3.5 to 5.5 mg of microparticles were dispersed in a solution of collagenase in TESCA buffer (50 mM of N-Tris(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, 0.36 mM of calcium chloride $CaCl_2$, pH = 7.4). It was then incubated at 37 °C under slight agitation for 24 h. The remaining particles were then rinsed 3 times with DI water and finally freeze-dried, and weighed. This mass was then compared to the initial mass of the samples to determine the percent degradation of the microparticles.

Degradation of the Microparticles in a MMP-2 Solution. To study MMP-2 degradation, an assay buffer made of 50 mM of Tris-HCl, 10 mM of calcium chloride, 150 mM of sodium chloride, and 0.1 mM of Brij-35 was prepared to activate the as-received MMP-2. Pro-MMP-2 was activated by diluting it to a concentration of 100 μg/mL in the assay buffer, adding 4-aminophenylmercuric acetate to it at a final concentration of 1 mM and incubating it at 37 °C for 1 h. The activated MMP-2 was then diluted in the assay buffer, and this solution was then added to 3.5 to 5.5 mg of microparticles. The particles were then incubated with the activated MMP-2 at 37 °C under slight agitation for 24 h. After incubation, the remaining particles were rinsed 3 times with DI water, and finally freeze-dried and weighed. This mass was then compared to the initial weight of the samples to determine the percent degradation of the microparticles.

3. RESULTS AND DISCUSSION

3.1. Synthesis of the Bis-poly(ethylene glycol) Acrylate Gly-Leu-Lys Conjugate. One method by which to impart enzyme sensitivity to a carrier is to incorporate a specific peptide sequence into the PEGDA backbone. The peptide used here is a three amino acid peptide sequence: Gly-Leu-Lys. This peptide was chosen because the Gly-Leu bond can be cleaved by collagenase, while the Lys amino acid was selected because it provides an additional amine for incorporation within the PEGDA macromer. Scheme 1 shows the coupling chemistry between the peptide and the PEG. The precursor chosen is an acrylate-poly(ethylene glycol)-succinimidyl valerate (Acr-PEG-SVA). The coupling results presented here were studied with a 5000 g/mol precursor (Acr-PEG(5000)-SVA) and a 2000 g/mol precursor (Acr-PEG(2000)-SVA). The succinimidyl valerate group can easily react with one of the two free amines on the peptide chains. With a ratio of 2:1 for the PEG precursor, two PEG chain are coupled on each peptide molecule, to obtain a bis-poly-(ethylene glycol) acrylate Gly-Leu-Lys conjugate.

Scheme 1. Coupling Chemistry between the Gly-Leu-Lys Peptide and the PEG Precursor



The covalent coupling between the peptide and the Acr-PEG(5000)-SVA was confirmed by FTIR, and by two chemical tests: the ninhydrin test and the biuret test, and by GPC-MALS. The FTIR spectra are presented in Figure 1. The first noticeable observation is the appearance in the bis-poly-(ethylene glycol) acrylate Gly-Leu-Lys of a large band between 1630 and 1680 cm^{-1}, which can be attributed to the stretching vibration of the C═O bound in the amide groups. At the same time, the bands between 1775 and 1830 cm^{-1}, which were present in the Acr-PEG(5000)-SVA spectrum, are absent in the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate spectrum. Those bands were attributed to the stretching vibration of the C═O bonds of the succinimide group, which disappears after the reaction.

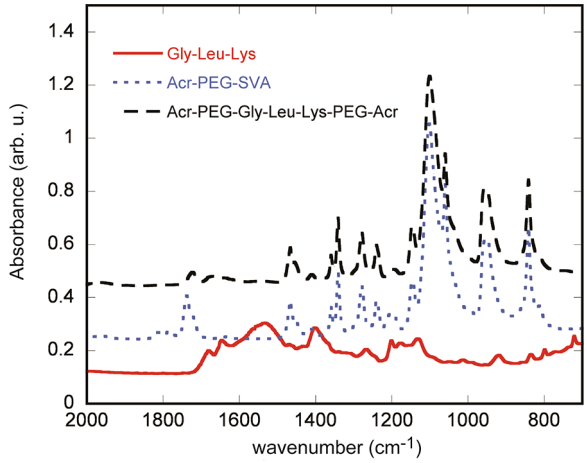


Figure 1. Fourier transform infrared spectra of the Gly-Leu-Lys peptide (solid red line), the Acr-PEG(5000)-SVA precursor (dotted line), and the bis-poly(ethylene glycol) acrylate peptide conjugate (dashed line).

A biuret and a ninhydrin test were also performed to confirm the covalent conjugation of the peptide to the Acr-PEG(5000)-SVA, and the results are presented in Supporting Information Figure S1. The biuret test is a chemical test used to detect

peptide bonds. In the biuret test, the purple color appeared only for the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate indicating the presence of amide bonds, which were not present in the PEGDA alone. The ninhydrin test is a commonly used chemical test to detect the presence of amine groups. The yellow coloration of the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate with ninhydrin does not indicate the presence of primary amines, contrary to the test with peptide alone where the solution becomes purple, result of a positive ninhydrin test. The bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate contained amide functionality and did not present any primary amine function, revealing the successful incorporation of the peptide into the polymer chain.

Finally, the coupling of the Acr-PEG(5000)-SVA and the Acr-PEG(2000)-SVA with the Gly-Leu-Lys peptide was confirmed by gel permeation chromatography coupled with multiangle light scattering (GPC-MALS) (Table 1). While the precursor Acr-PEG(5000)-SVA presented one peak centered on 4400 g/mol, the product obtained, bis-poly(ethylene glycol) (5000) acrylate Gly-Leu-Lys, presented two peaks, where 81% of the mass was described by a peak centered at 10 500 g/mol, which corresponds to the molecular weight of the bis-poly(ethylene glycol)(5000) acrylate Gly-Leu-Lys conjugate, and 13.4% of the mass was described by a peak centered at 5600 g/mol, which corresponds to a peptide that reacted with only one Acr-PEG(5000)-SVA. The same phenomenon is observed for the coupling of the Acr-PEG(2000)-SVA and the Gly-Leu-Lys. An additional 5% for the Acr-PEG(2000)-SVA showed up in a high molecular weight peak, that was attributed to aggregated material. These results confirm the incroporation of the peptide to the PEG precursors to obtain a bis-poly(ethylene gycol) acrylate Gly-Leu-Lys.

Table 1. GPC-MALS Results for the Acr-PEG-SVA and the Bis-poly(ethylene glycol) Acrylate Gly-Leu-Lys Conjugate

	fraction 1		fraction 2	
	MW (g/mol)	mass fraction (%)	MW (g/mol)	mass fraction (%)
Acr-PEG(5000)-SVA	4400 (±9)	100	X	X
Acr-PEG(5000)-Gly-Leu-Lys-PEG(5000)-Acr	5600 (±17)	13.4	10500 (±210)	81
Acr-PEG(2000)-SVA	2300 (±57)	95	X	X
Acr-PEG(2000)-Gly-Leu-Lys-PEG(2000)-Acr	2650 (±74)	29	4720 (±76)	71

3.2. PEGDA Microparticles Synthesis. Before exploring the possibility to make enzyme-sensitive particles, a reproducible method to make PEG microparticles with sizes between 1 and 10 μm from high molecular weight macromers is needed. In this study, PEG microparticles were synthesized using an inverse emulsion method. The synthesis was performed in silicone oils with a range of viscosities, a water phase, and the polymerization was photoinitiated. In all cases the photoinitiator, 2,2-dimethoxy-2-phenylacetophenone (DMPA) was dissolved in the silicone oil and was then added to a concentrated aqueous solution of PEGDA. In the remainder of this paper, the notation PEGDA-X will be used, where X refers to the molecular weight of the PEGDA macro-monomer used in g/mol. In this study, molecular weights of PEGDA from 700 up to 10000 g/mol were used because the further incorporation of large biologically relevant molecules, as peptides or proteins, requires the use of high molecular weight polymers.

Emulsion based polymerization to make PEG microparticles has been previously presented by other groups using low molecular weight PEGDAs (MW = 575 or 700 g/mol), which are liquid at ambient temperature.[48−50] In these studies, the authors showed that the use of silicone oil instead of mineral oil resulted in the formation of smaller microparticles, and that the more viscous the oil, the smaller the particles are. Therefore, we initially tested the protocol with PEGDA-700, and silicone oils with a kinematic viscosity of 1000, 5000, or 10000 cSt. For PEGDA-700, only 2 min under a 365 nm lamp was necessary to form microspheres with the lowest viscosity silicone oil (1000 cSt), and microparticles with an average diameter of 4.38 μm were obtained (Figure 2a and Table 2). The use of higher viscosity silicone oil (5000 or 10000 cSt) did not lead to smaller particles. However, this can be attributed to the fact that the more viscous oils did not produce a stable emulsion.

Above a molecular weight of 1000 g/mol PEGDA is solid at ambient temperature, requiring the development of modified processes to form microparticles using higher molecular weight precursors via inverse emulsion methods. Previous groups,

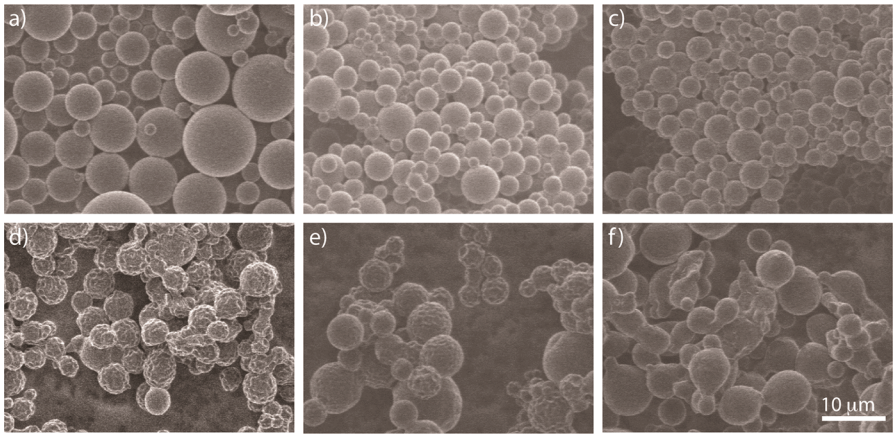


Figure 2. Scanning electron micrographs of microparticles made from different molecular weight PEGDA. (a) MW = 700 g/mol, (b) MW = 2000 g/mol, (c) MW = 3400 g/mol, (d) MW = 5000 g/mol, (e) MW = 6000 g/mol, (f) MW = 10000 g/mol. Scale bar is 10 μm and is the same for all images.

Table 2. Table Summarizing the Unswollen and Swollen Sizes, and the Swelling Ratio, of the Microparticles Obtained with the Different Molecular Weight PEGDA[a]

macromer	concn. in water (in wt %)	mean diam. of the freeze-dried microparticles (μm)	mean aerodynamic diam. of the microparticles (μm)	mean diam. of the microparticles in water (μm)	swelling ratio
PEGDA700	pure (liquid)	4.38 ± 2.49	4.64 ± 2.64	7.96 ± 3.20	6.00
PEGDA2000	70%	2.83 ± 1.19	2.99 ± 1.26	6.83 ± 2.38	14.06
PEGDA3400	70%	2.81 ± 0.92	2.97 ± 0.97	6.14 ± 2.07	10.43
PEGDA5000	60%	3.94 ± 1.60	4.17 ± 1.69	8.74 ± 3.61	10.92
PEGDA6000	60%	3.94 ± 1.70	4.17 ± 1.80	8.06 ± 3.07	8.56
PEGDA10 000	60%	3.87 ± 1.75	4.10 ± 1.85	7.82 ± 4.25	8.25
PEG(5000)-Gly-Leu-Lys	70%	6.74 ± 2.93	7.13 ± 3.10	13.03 ± 5.24	7.23
PEG(2000)-Gly-Leu-Lys	70%	3.94 ± 1.84	4.17 ± 1.95	8.09 ± 3.72	8.66

[a]The swelling ratio is the ratio of the mean volume of the particles in water to the mean volume of the freeze-dried particles.

including Olabisi et al. and Franco et al. were able to obtain large particles by forming emulsions with low concentrations of PEGDA in water and cross-linking it with the use of one or two photoinitiators.[51,52] Here, we instead used a high concentration of PEGDA in water to mimic the high concentration of polymer present in the liquid polymer form (e.g PEGDA-700). By this method, spherical microparticles with a mean diameter between 2.8 and 4 μm were obtained after freeze-drying for PEGDAs with molecular weights for the starting material between 2000 and 10 000 g/mol (Figure 2 and Table 2). To obtain these microparticles, the cross-linking time of 2 min used for PEGDA-700 was not sufficient, instead 30 min under the 365 nm lamp was necessary in order to obtain microparticles. Since 30 min under the UV lamp is required to have stable cross-linked microparticles, the emulsion must remain stable during this time period. We prepared different emulsions with solutions of different concentrations of PEGDA-3400 in water (10%, 30%, 50%, and 70% wt %) via vortex mixing and monitored their stability over time. After 2 min, the emulsion based on the 10 wt % PEGDA solution became unstable, producing large droplets of water. For the emulsion based on the 30 wt % PEGDA solution, the emulsion became quickly unstable, with water droplets appearing after 1 h. However, the emulsion based on the 50 wt % PEGDA solution appeared to remain stable for up to 24 h, after which a phase separation was observed. The emulsion based on the highest concentration of PEGDA-3400 (70 wt %) remained completely stable after 24 h. Figure 3 provides optical and electron micrographs of the resultant swollen and freeze-dried particles, respectively. The particles obtained from the less stable 30 wt % PEGDA-3400–based emulsion (Figure 3a, c) are larger and nonspherical, contrary to what was obtained from the stable emulsion based on the 70 wt % PEGDA-3400 solution (Figure 3b, d). The macromer concentration also plays an important role in controlling the topography of the particles. Figure 2d shows PEGDA-5000 particles that are spherical but present a rough surface. However, this "dimpled" morphology can be smoothed by varying the concentration of the polymer used. Figure 4 presents SEM images of PEGDA-5000 particles made from solution with concentration of 60%, 65%, and 70% in water. It is important to note that the 70% PEGDA-5000 solution is close to the solubility limit, and this solution may not be fully dissolved. Figure 4 shows that higher macromer concentrations result in the formation of particles with smoother surfaces. This provides the ability to tune the surface roughness in order to have enhanced delivery performance.[56] Finally, we also found that we could decrease the polydispersity

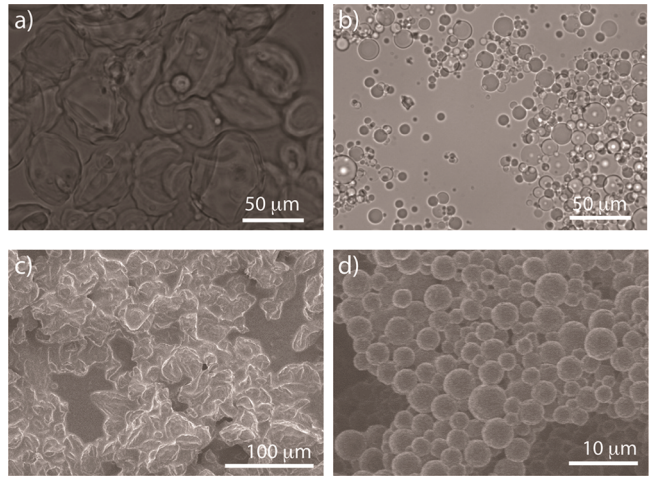


Figure 3. Optical micrographs of swollen particles obtained from PEGDA-3400 at (a) 30% and (b) 70% in water and corresponding electron micrographs of freeze-dried particles in parts c and d, respectively.

of the PEG microparticles by increasing the vortexing time of the emulsion (Supporting Information Figure S2a).

After synthesizing the microparticles from different molecular weight macromers, their physical properties were analyzed (Table 2). It is worth noting, that the amount of photo-cross-linker, DMPA, was held constant (50 mg) for the results shown in Table 2. Table 2 shows the swelling ratio of the particles, which is the ratio of their volume after swelling in water for a few hours to their volume after freeze drying (although we noticed that complete swelling of the particles occurred in under a minute). It is worth noting that the swelling of the particles is the same in both water and PBS. It shows that for the same concentration of PEGDA in water, the swelling ratio decreases with increasing molecular weight of the starting macromer. One possible explanation for this behavior can be attributed to increasing molecular entanglements when the precursor chains are longer. These results also show that for each molecular weight tested, the particles have an appropriate size for pulmonary delivery via inhalation, since the optimum aerodynamic diameter for that is between 0.5 and 5 μm. The diameter of the dried particles was also measured by the use of an Aerosizer (TSI PSD 3603). The results, presented in Supporting Information Table S1, showed that those particles can be aerosolized and are not strongly aggregated. These

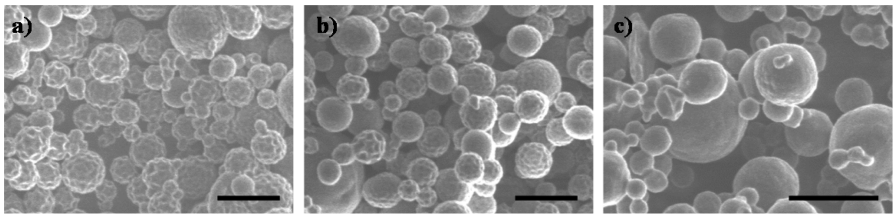


Figure 4. Scanning electron micrographs of PEGDA-5000 microparticles synthesized from solution of polymer in water with concentration of (a) 60%, (b) 65%, and (c) 70%. Scale bars are 10 μm.

particles are also large enough in their swollen state (>6 μm) to avoid a rapid macrophage clearance.[16−18]

Another critical parameter for the synthesis of these microparticles is the cross-linker DMPA, both the quantity used and its dispersion phase. The phase in which the DMPA resides determines whether the polymerization occurs via an emulsion or suspension mechanism. As outlined previously, one of the benefits of an emulsion method is the production of monodisperse particles. For the case of suspension based polymerization of PEG microparticles, the photoinitiator DMPA can be dissolved in PEGDA-700 and in low concentration aqueous PEGDA solutions, as demonstrated by Flake et al.[46] However, DMPA has a low solubility in the concentrated PEGDA solutions that are used in the present work limiting this mechanism for the synthesis of microparticles from high molecular weight PEGDA macro-monomers. DMPA does, however, have a high solubility in silicone oil, leading to subsequent polymerization via the inverse emulsion mechanism described here. Though we propose that an emulsion mechanism is taking place in this study, it is of course possible that some of the initiator is in the aqueous PEGDA phase and that some particles form via suspension polymerization. But, due to the low solubility of the DMPA in the concentrated aqueous PEGDA solution, we propose that the emulsion mechanism dominates the formation of the PEGDA microparticles. The quantity of DMPA used is also important in the synthesis of the microparticles. Supporting Information Figure S2b shows that for concentrations of DMPA lower than 5 wt %, only large particles, with a mean diameter around 45 μm were obtained. When the quantity of DMPA was higher than 5 wt %, the mean diameter of the particles decreased to between 2.8 and 4 μm. Additionally, the quantity of DMPA influenced the yield of the synthesis. The highest yield was obtained by using DMPA as high as 50 wt %, which we attribute to providing a higher number of nucleation points when using a higher concentration of photoinitiator. This result also points to the inverse emulsion mechanism as being the dominant mechanism. At low DMPA concentrations it is likely that a higher relative fraction of DMPA is present in the aqueous phase, however at higher DMPA concentration the photoinitiator will be present in a much higher fraction in the oil phase, due to its high solubility in the oil phase compared with the aqueous phase. Supporting Information Figure S2b also reveals that for concentrations of DMPA between 5 and 50 wt % that the mean diameter of the particles does not change much.

3.3. Enzyme-Sensitive Microparticles Synthesis. The microparticle synthesis process described here is particularly versatile because it allows the precursor macromer to be high molecular weight polymer chains, providing a process that can readily incorporate large biologically relevant molecules, such as peptide or proteins. Here, the microparticle synthesis process described above to obtain PEGDA microparticles was extended to produce particles sensitive to matrix metalloproteinases (MMPs), by using the bis-poly(ethylene glycol) acrylate Gly-Leu-Lys conjugate, the synthesis of which is described above.

Microparticles formed from two different molecular weight bis-poly(ethylene gycol) acrylate Gly-Leu-Lys conjugates (Acr-PEG(2000)-Gly-Leu-Lys-PEG(2000)-Acr and Acr-PEG(5000)-Gly-Leu-Lys-PEG(5000)-Acr) were synthesized using the inverse emulsion process as described earlier. The concentration of the solution of bis-poly(ethylene gycol) acrylate peptide in water was 70 wt % for both molecular weights tested, higher than the concentration used for the PEGDA-10000 (60%). This increase in polymer solution concentration was possible because the peptide allowed for increased solubility of the macromer in water. The particles obtained by this process, which will further be called PEG(2000)-Gly-Leu-Lys and PEG(5000)-Gly-Leu-Lys microparticles, are presented in Figure 5. Their physical properties are presented in Table 2. Here, again the synthesized particles fulfill the size requirements for lung delivery by inhalation.

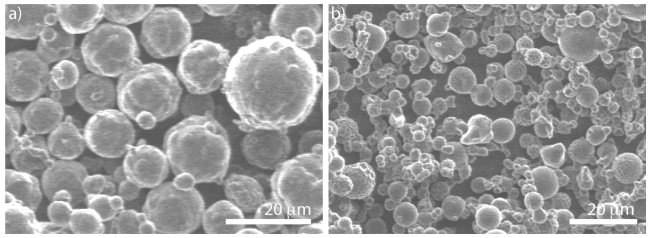


Figure 5. Scanning electron micrographs of microparticles synthesized from (a) PEG(5000)-Gly-Leu-Lys and (b) PEG(2000)-Gly-Leu-Lys.

3.4. Degradation of the Enzyme-Sensitive Microparticles. The enzymatic degradation of the microparticles was studied, by exposing the microparticles to a solution of MMP-1 (collagenase) enzymes. The degradation results for microparticles with the incorporated peptide were compared to microparticles made from the high molecular weight PEGDA:PEGDA-10000. The degradation profiles are presented in Figure 6. The PEG(5000)-Gly-Leu-Lys microparticles underwent some degradation after 24 h in a collagenase solution with collagenase concentrations as low as 2 μg/mL. At collagenase concentrations higher than 6 μg/mL, all the PEG(5000)-Gly-Leu-Lys particles were completely degraded after 24 h. In comparison, the PEGDA-10000 microparticles did not show increased degradation in a collagenase solution (up to 60 μg/mL) compared to PBS (data not shown). However, it is anticipated that over longer time scales the PEGDA-10000 would undergo degradation via hydrolysis. The peptide, if present in the polymer sequence, is hydrolyzed by the collagenase enzyme, hence the PEG-peptide particles were degraded while the PEGDA-10000 particles that do not include

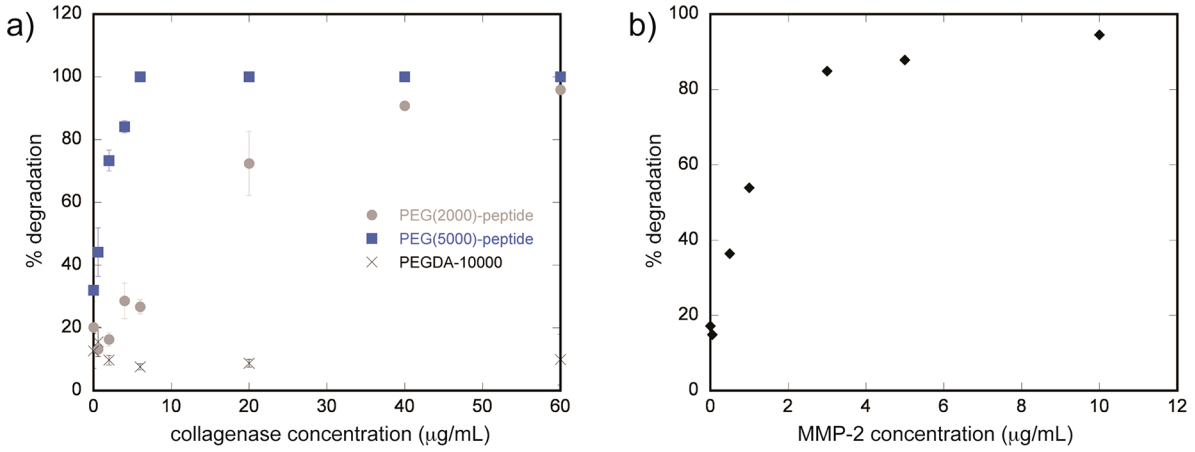


Figure 6. Degradation of (a) PEG(5000)-Gly-Leu-Lys microparticles (blue square), PEG(2000)-Gly-Leu-Lys (gray circle), and PEGDA-10000 microparticles (x) with different concentrations of collagenase in solution, after 24 h. (b) Degradation of the PEG(5000)-Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys microparticles with different concentrations of MMP-2, after 24 h.

the peptide sequence were not. The PEG(2000)-Gly-Leu-Lys microparticles required a larger concentration of enzyme for degradation. The complete degradation of the PEG(2000)-Gly-Leu-Lys microparticles did not occur before MMP-1 concentrations reached 60 μg/mL. This can be explained by the fact that the shorter chains would be expected to cause a smaller mesh size, limiting enzyme diffusion within the particles. The degradation would only be able to occur from the surface of the particles, while for the PEG(5000)-Gly-Leu-Lys microparticles, the mesh size is large enough for the enzyme to diffuse into the particles, allowing for more rapid degradation.

The degradation of the microparticles can also be tuned by changing the length of the peptide sequence in the backbone of the polymer. Although the degradation results were good with the PEG(5000)-Gly-Leu-Lys, we wanted to see if a different sequence would allow for an increased sensitivity to lower enzyme concentrations. In order to do that, the Gly-Leu-Lys peptide was replaced by a nine amino acids peptide: Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys.[57] While the PEG(5000)-Gly-Leu-Lys microparticles were completely degraded with collagenase for concentrations higher than 6 μg/mL, the PEG(5000)-Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys microparticles were completely degraded with a concentration of collagenase of 0.6 μg/mL. This increase in sensitivity can be explained by the fact that the longer peptide can have a better conformation and a better affinity for the collagenase enzyme, and also by the possible increase mesh size of the hydrogel microparticles. Additionally, the incorporation of this longer peptide sequence allows the particles to be degraded by MMP-2, which is overexpressed in lung cancer. The hypothesis here is that a three amino acid peptide does not have a good affinity with the MMP-2's large binding site, whereas the nine amino acids peptide would adopt the right conformation to interact with the enzyme. Finally, the PEG(5000)-Gly-Leu-Lys microparticles were not degraded by MMP-2 enzyme even at a concentration of 10 μg/mL. By changing the peptide chain to the Gly-Pro-Gln-Gly-Ile-Phe-Gly-Gln-Lys peptide, 85% of the microparticles were degraded with a MMP-2 concentration of 3 μg/mL, as presented in Figure 6.

4. CONCLUSION

Finally, in this work we have shown that by choosing the right concentration of the aqueous solution of a PEGDA macromonomer, it is possible to obtain small PEG microspheres by an inverse emulsion technique. This technique provides a versatile method to form monodisperse PEG-based microparticles from a wide range of molecular weights, ranging from liquid low molecular weight PEGDAs to solid high molecular weight PEGDAs as starting materials, including precursors that incorporate biomolecules. Particles with a diameter around 3 μm have been obtained for PEGDA macro-monomers with molecular weights between 2000 and 10000 g/mol. Moreover, this is a simple technique that is easy to implement in any lab, with common bench equipment. Lastly, another key aspect of this procedure is that no surfactant or any other polymers have to be added, making this type of synthesis a good candidate for biomedical applications. These particles can also be rendered enzyme-responsive, by incorporating a peptide sequence in the polymer backbone. The inverse emulsion technique presented here produced microparticles of the appropriate size to be used for pulmonary delivery by inhalation, and can be degraded in the presence of low amount of MMP. Further work will be performed to confirm the safety and biocompatibility of these particles as well as to study their mucoadhesive properties. Their degradation properties can be linked to their physical properties. Lastly, this method could be implemented with a wide variery of peptides or biomolecules to develop microparticles for applications in drug delivery. In future drug release applications, we anticipate that the release of the drug molecule(s), would happen coincident with the enzymatic degradation of the particles.

■ ASSOCIATED CONTENT

Supporting Information

Ninhydrin test, biuret test, measurements of the size of PEGDA-3400 microparticles versus the duration of vortexing and the quantity of DMPA, and dried particles size measurements performed with a TSI PSD 3603 particle size distribution analyzer. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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