MMP7-Responsive Nanocarriers for Intelligent Drug Delivery in Tumors

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Abstract: Intelligent drug delivery has been one of the most successful ways of successful cancer treatment. Noticing the significantly high MMP-7 concentrations around tumor regions, intelligent drug nanocarrier was assembled by PEG_{2k}-Peptide-$PLGA_{2k}$ polymers in this research. The MMP-7 responsiveness towards the nanocarriers was then evaluated to verify their potential of achieving effective tumor-region drug localization.

1 Introduction

Ineffective therapeutic targeting has been one of the main reasons for the negligible advancement on overall cancer survival for a long time. However, intelligent drug delivery, aiming at the signature of micro-environment around specific cancer cells to enable the effective release of cancer drugs, has been proved useful in tackling this problem. A common signature of cancer cells is low pH and oxygen level due to the high demand of oxygen which leads to anaerobic respiration [3]. Meanwhile, more recent studies have shown that matrix metalloproteinases (MMPs), enzymes that are capable of degrading certain kinds of extracellular matrix proteins, can be a significant biomarker for cancer environments [8]. By assembling nanocarriers using synthesized polymers containing MMP-cleavable peptide, its size and chemical features can be altered by MMP to achieve intelligent drug delivery. [10]. It has been found that MMP-7 concentrations has remarkably high concentration in tumors [5], so a drug delivery system with nanocarriers formed by polymers with structure PEG-(MMP-7-peptide)-PLGA can be effective in its treatment.

2 Keywords

MMP-7; Intelligent drug delivery; Macromolecule cleavage, Enhanced permeability and retention effect (EPR effect).

3 Materials and Methods

Synthesis of PEG_{2k}-Peptide. 12.1 mg PEG_{2k}-MAL (JenKem Technology) and 5 mg MMP-7 cleavable peptide (NH_2-VPSLYSGCG-COOH, GL Biochem, MW=995 Da) were added to 2 ml anhydrous dimethylformamide (DMF, Energy Chemical) and 0.19 ml triethanolamine (TEA, Sinopharm Chemical Reagent). The reaction took place for 24 h under argon gas flow at room temperature. The product was purified from excess MMP-7 cleavable peptide against diH_2O (Advantage A10, Milli-Q) for 28 h using dialysis tubing of MWCO = 3.5 kDa (SnakeSkin, Thermo Scientific). The liquid was then pre-frozen in -80°C freezer (Forma 900 Series, Thermo Scientific) for 1 h before sending to freeze-dry (VaCo 5, Zirbus Technology) for 24 h.

Synthesis of PEG_{2k}-Peptide-$PLGA_{2k}$. 13.7 mg PEG_{2k}-Peptide (previously described) and 27.4 mg $PLGA_{2k}$-COOH (Jinan Daigang Biomaterial) were added to 5.2 mg HATU (Energy Chemical), 2.2 μl N,N-Diisopropylethylamine (DIPEA, 1.7 mg, Energy Chemical), and 2 ml anhydrous dimethyl sulfoxide (DMSO, Energy Chemical). The reaction took place for 36 h under argon gas flow at room temperature. The product was purified from excess $PLGA_{2k}$ against DMF (Sinopharm Chemical Reagent) for 18 h using dialysis tubing of MWCO = 3.5 kDa, then DMF and other organic solvents were removed by dialysis against diH_2O using the same type of dialysis tubing for 8 h. The remaining liquid was then pre-frozen in -80°C freezer for 1 h and sent to freeze-dry for 24h.

Self-assembly of Drug Nanocarriers and Size Measurement by DLS. 2.1 mg PEG_{2k}-Peptide-$PLGA_{2k}$ was dissolved into DMF at 1 mg/ml. 500 μl of the solution was transferred to the dialysis tubing of MWCO = 3.5 kDa. 250 μl and 125 μl of the solution correspondingly with 250 μl and 375 μl diH_2O were transferred to two other dialysis tubings of the same type. The three concentrations (1 mg/ml, 0.5 mg/ml, and 0.25 mg/ml) were dialyzed against diH_2O for 18 hours. Hydrodynamic radii for nanocarriers formed under three different concentrations were measured using dynamic

light scattering (Zetasizer Nano-ZS, Malvern) after passing through 0.22 μm syringe filter (SF-13NL22-0100, Anaqur Chemical Supply).

Preparation for MMP-7 Responsiveness Assessment. MMP-7 active human enzyme (1.9 mg/ml, 19.13 kDa, enz-271, Prospec) was stored in additives of 10 mM HEPES, 5 mM $CaCl_2$, and 150 mM NaCl. 24 mg HEPES (Thermo Fisher Scientific), 5.5 mg $CaCl_2$ (Sinopharm Chemical Reagent), and 87.7 mg NaCl (Sinopharm Chemical Reagent) were added to 10 ml diH_2O to prepare enzyme storage additive for later usage. For the proper environment of MMP-7's response assessment, 1.60 g NaCl, 0.04 g KCl (Sinopharm Chemical Reagent), 0.04 g KH_2PO_4 (Sinopharm Chemical Reagent), 0.68 g $Na_2HPO_4 \cdot 12H_2O$ (Sinopharm Chemical Reagent), and 1.4 mg $ZnCl_2$ (Sinopharm Chemical Reagent) were added to 100 ml di H_2O [6]. The buffer solution was filtered by 0.22 μm syringe filter and stored for later use.

Assessment of the MMP-7 Responsiveness of PEG_{2k}-Peptide-$PLGA_{2k}$ Nanocarriers. Research has shown that 50nM and 1nM are the general MMP-7 concentrations in human tumor regions and healthy tissues correspondingly [7]. 250 μl of the buffer solution and 250 μl of the solution containing nanocarriers made from 0.5 mg/ml PEG_{2k}-Peptide-$PLGA_{2k}$ were added to nine tubes. Among the nine tubes, 10 μl 1 μg/ml MMP-7 (50 nM) solution was added to three of them, 10 μl 1 μg/ml MMP-7 (1 nM) solution was added to three other, and 10 μl additive solution was added to the remaining three tubes as control. The tubes were sealed and placed in temperature-controlled shaker (Taicang Hualida Laboratory Equipment). At 0 h, 2 h, 4 h, 6 h, 10 h, 24 h, 40 μl solution was collected from each tube and diluted to 100 μl. The samples were then measured three runs each on the DLS zetasizer.

Scheme 1. Synthetic Scheme for Synthesis of PEG_{2k}-Peptide-$PLGA_{2k}$.

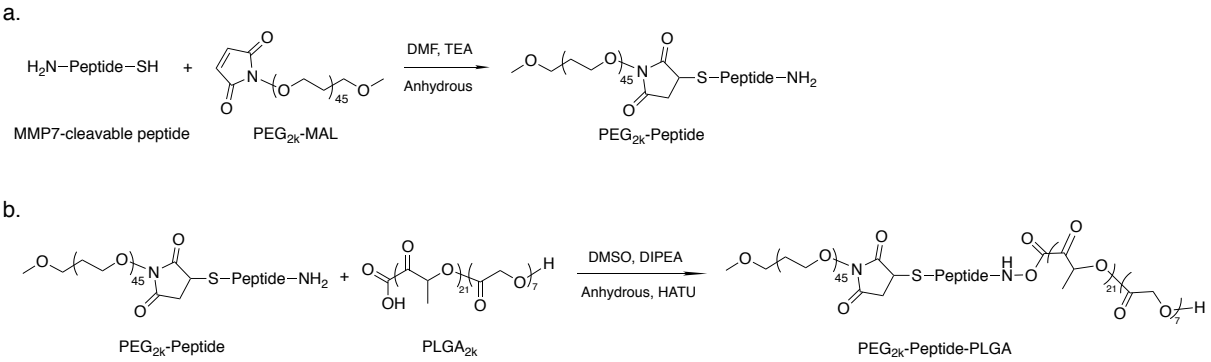
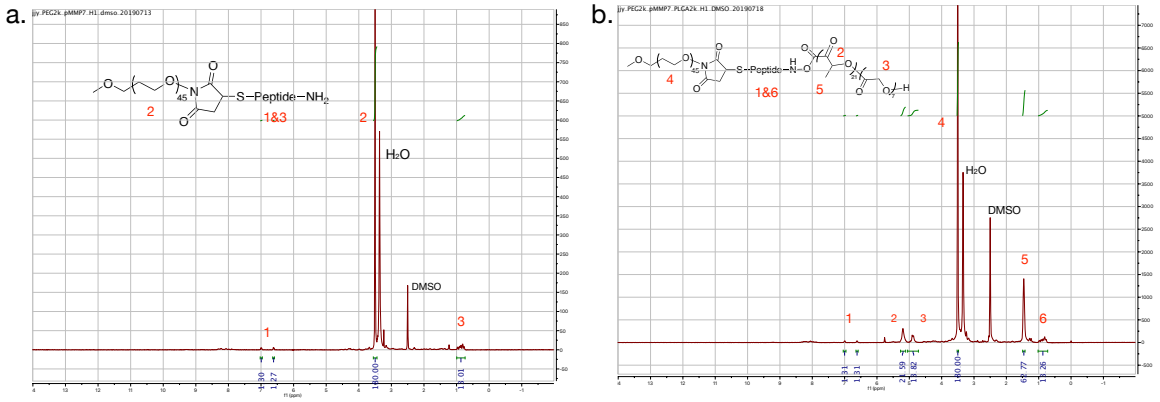


Figure 1. ^{1}H NMR Spectroscopy for PEG_{2k}-Peptide and PEG_{2k}-Peptide-$PLGA_{2k}$.



4 Results

Synthesis of PEG_{2k}-Peptide. Using the synthetic scheme in **Scheme 1.a**, 13.7 mg PEG_{2k}-Peptide was obtained after the freeze-drying process. 4.4 mg of the product was dissolved into 500 μl DMSO-d_6 (Sigma-Aldrich, 99.96 atom % D, contains 0.03 % (v/v) TMS) and sent to ^{1}H NMR

(400 MHz, Oxford NMR AS400). The ^{1}H NMR spectroscopy can be found in **Figure 1.a.**. After opening the file with MestReNova, the baseline was auto-corrected and the curve was shifted according to the reference of DMSO-d_6 at 2.50 ppm [2]. For verification, the peak of water should be found at 3.33 ppm [2] and the peak of TMS at around 0.00 ppm. Previous research has shown that the peak of the PEG backbone is at 3.51 ppm [1]. There were 45 backbone blocks (CH_2CH_2O) in PEG_{2k} and four hydrogen atoms in each block. Therefore, the peak at around 3.51 ppm was integrated and its value set to 180.00. After that, two small peaks between 6.50 ppm and 7.00 ppm and a large peak between 0.50 ppm and 1.00 ppm, belonging to the hydrogen atoms in the peptide chain, are integrated, giving values of 1.30, 1.27, and 13.01 correspondingly. The ^{1}H NMR spectroscopy of MMP-7 cleavable peptide can be found in **Supporting Information 1.**.

Synthesis of PEG_{2k}-Peptide-$PLGA_{2k}$. Using the synthetic scheme in **Scheme 1.b**, 11.4 mg PEG_{2k}-Peptide-$PLGA_{2k}$ was obtained. 3.5 mg of the product was then dissolved into 500 μl DMSO-d_6 and sent to ^{1}H NMR. The ^{1}H NMR spectroscopy can be found in **Figure 1.b.**. After opening the file with MestReNova, baseline was auto-corrected and the curve was shifted according to the reference of DMSO-d_6 at 2.50 ppm. For verification, the peak of water should be found at 3.33 ppm and the peak of TMS at around 0.00 ppm. The peak at around 3.51 ppm was integrated and set to 180.00 for reference. For verification, the three peptide peaks that were previously mentioned were integrated and the values matched. There have been three peaks found for PLGA: at 1.46 ppm, 4.91 ppm, and 5.19 ppm, which corresponds to the methyl group on the lactic acid, the methylene group on the glycolic acid, and the methine group on the lactic acid [11]. Integrating the three peaks, values were found matching with the polymerization number: 63 hydrogens connected to 21 methyl carbons, 14 hydrogens connected to 7 methylene carbons, and 21 hydrogens connected to 21 methine carbons. Accordingly, this proved the successful linkage between PEG, peptide, and PLGA.

Self-assembly of Drug Nanocarriers and Size Measurement by DLS. Hydrodynamic radii were measured by DLS for the three groups. Each group contained three runs and the results can be found in **Table 1.**. 0.5 mg/ml nanocarriers were chosen for later steps due to their most proper hydrodynamic radii.

Table 1. Hydrodynamic Radii for Nanocarriers Assembled under Different Concentrations.

	Intensity Mean (nm)		**Number Mean (nm)**		**PDI**	
	Mean	SD	Mean	SD	Mean	SD
1 mg/ml	377.57	15.57	263.83	30.29	0.106	0.071
0.5 mg/ml	291.53	9.92	115.53	19.46	0.194	0.014
0.25 mg/ml	288.30	23.25	98.83	37.26	0.189	0.028

Assessment of the MMP-7 Responsiveness of PEG_{2k}-Peptide-$PLGA_{2k}$ Nanocarriers. The change for hydrodynamic radii over time for the three groups can be found in **Figure 2.a.**. The red curve with label "E-50", the green curve with label "E-1", and the blue curve with label "C" corresponds to the 50 nM MMP-7 group, the 1 nM MMP-7 group, and the control group. Raw comparison number mean curve at 24 h generated by the DLS zetasizer can be found in **Supporting Information 2.** and hydrodynamic radii change raw data can be found in **Supporting Information 3.**.

5 Discussion

Self-assembly of drug nanocarriers and size measurement by DLS. From the data in **Table 1.**, the hydrodynamic radii of nanocarriers made from 0.5 mg/ml PEG_{2k}-Peptide-$PLGA_{2k}$ had the intensity mean of 291.53 nm and the number mean of 115.53 nm, with the standard deviation of 9.92 and 19.46 correspondingly. Among the three groups, radii of nanocarriers in the 0.5 mg/ml group were the best fit for MPP-7 responsiveness assessment and had the least SD value, indicating their stability among different runs. Therefore, 0.5 mg/ml group was chosen to continue with the enzyme responsiveness assessment.

Analyzing the MMP-7 Responsiveness Assessment Data. Based on the data in **Supporting Information 3.**, the control group started from 276.633 nm with the standard deviation of 6.716. From the data of the control group at 24 h, a slight decrease of 28.1% was noticed, indicating the possibility of minor hydrodynamic radii decrease under human body temperature and chemical environment. At 1 nM, the concentration of MMP-7 at non-cancer regions, a sharper decrease was noticed compared to the control group, namely

35.5% at 24 h. The difference showed that MMP-7, though at the concentration of healthy tissue, had a considerable effect on the hydrodynamic radii of the PEG_{2k}-Peptide-$PLGA_{2k}$ nanocarriers. At 50 nM, the concentration of MMP-7 around cancer cells, an unusual trend was noticed. Also starting at 276.633 nm, the 50 nM group had the radii decreased to 229.820 nm at 2 h, and then a slight increase of 25.1% from 4 h to 10 h. However, when the radii were measured again at 24 h, the value obtained was 0.8725 nm with SD of 0.2864. There was no further information collected to explain this unusual change, but a hypothesis can be proposed with a detailed explanation below.

Figure 2.a. Illustrative Diagram for the Change of Hydrodynamic Radii Over Time.

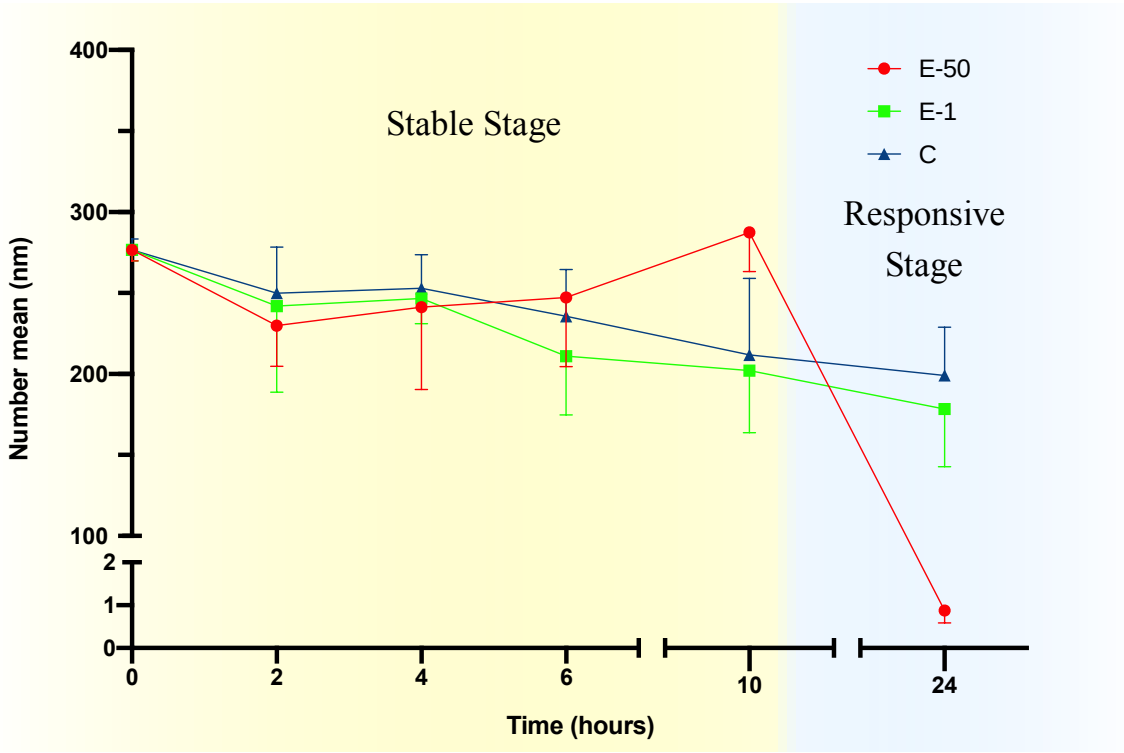
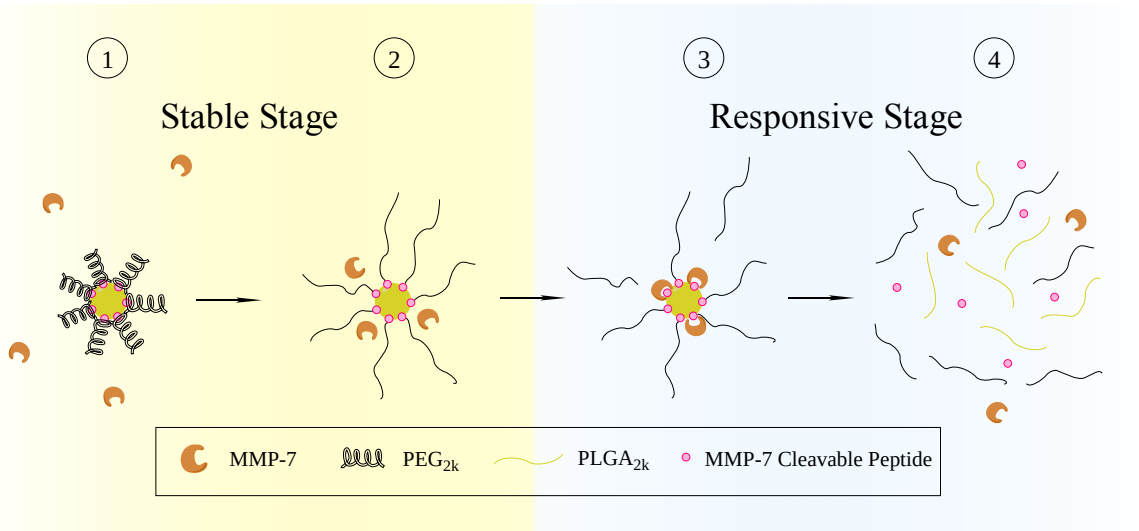


Figure 2.b. Illustrative Diagram of PEG_{2k}-Peptide-$PLGA_{2k}$ Nanocarriers Under 50 nM MMP-7 at Different Stage.



Proposed Hypothesis for the Unusual Change of the 50 nM Group from 2 h to 24 h. The abnormal increase of the hydrodynamic radii from 2 h to 10 h can be explained through the structural changes of the polyethylene backbones in PEG of the nanocarriers. Two forms for the polyethylene chain have been discovered by researchers: straight and helix form [4]. In **Figure 2.b.**, an illustrative diagram can be found explaining the different stage of the nanocarriers under 50 nM MMP-7. At 2 h, the nanocarriers are in stage ① . With the backbone curled up in helix form, most MMP-7 is blocked out. As time passes, under the gentle shake and 37°C temperature given by the shaker, the backbone is uncurled and it turns into straight form as shown in stage ② . This provides greater hydrodynamic radii, which match the data at 4 h, 6 h, and 10 h. In stage ③ , MMP-7 starts to bind to the cleavable peptide as the straight form of the backbone provides larger space. PEG starts to degrade as the linkage no longer exists. Finally, in stage ④ , as the PEG separates, the nanocarriers gradually loose their amphiphilicity and collapse. This proposed hypothesis is ideal for effective tumor localization. In the first 10 h, the nanocarriers have increased size and the drugs are kept safely inside. While the nanocarriers are circulated around the body, they accumulate at the tumor region due to the EPR effect: nanoparticles tend to accumulate in tumor tissues much more than they do in normal tissues [9]. After full circulations and when the nanocarriers settle down in cancer regions, the PEG backbone becomes straight which activates MMP-7 digestion, leading to the release of the cancer drugs right at the spot.

6 Conclusions

Intelligent drug delivery has played an important role in cancer treatment. In this research, PEG_{2k}-Peptide-$PLGA_{2k}$ was synthesized and verified through ^1H NMR spectroscopy. Hydrodynamic radii of the assembled nanocarriers were measured and the MMP-7 responsiveness was monitored at various time points via DLS. Satisfying experiment outcome and reasonable explanation indicate a great possibility for the PEG_{2k}-Peptide-$PLGA_{2k}$ nanocarriers to have effective tumor localization function. Further research around the hypothesis proposed in the discussion is very much worth conducting to provide evidence for the well-suitedness of the nanocarriers in cancer treatment.

7 Acknowledgements

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References

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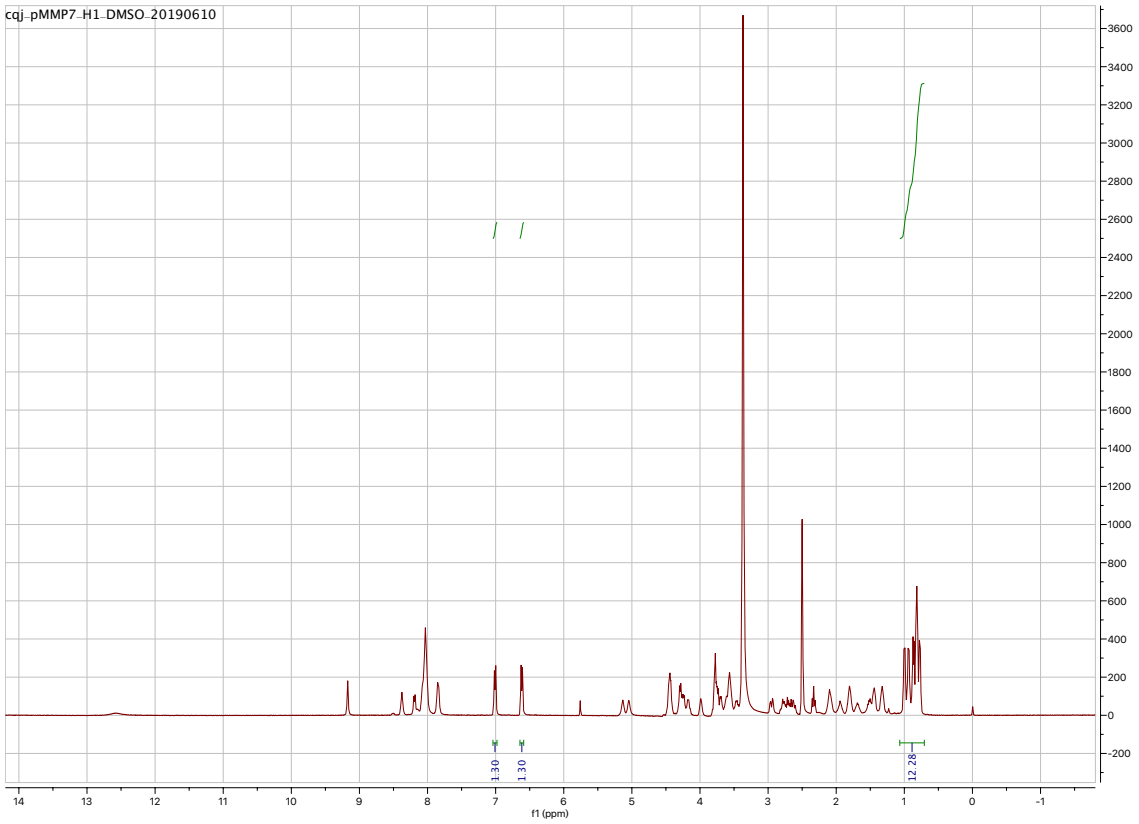
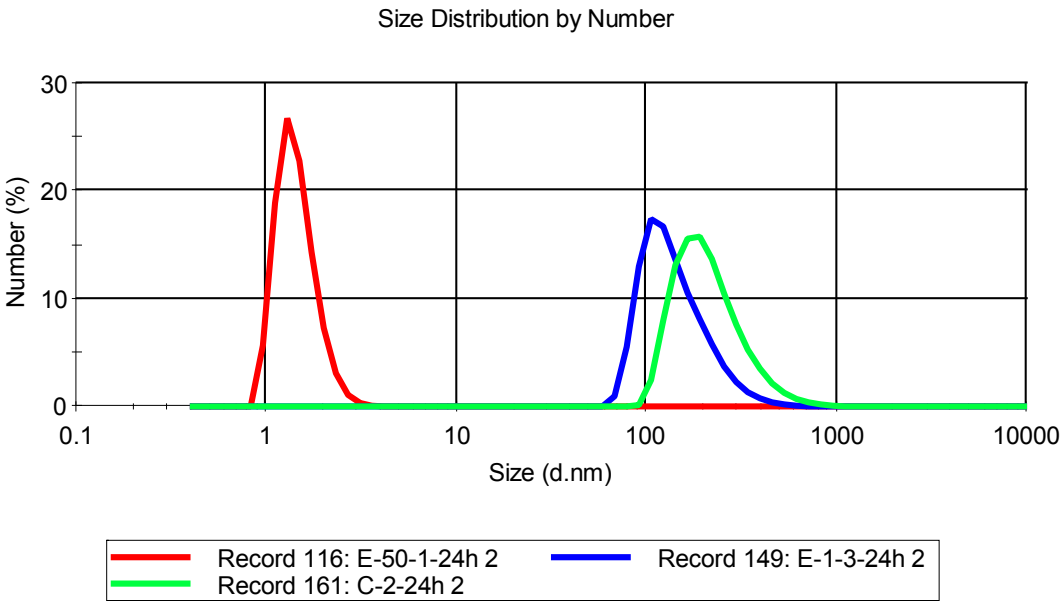
8 Supporting Information

Supporting Information 1. ^{1}H NMR Spectroscopy of MMP-7 Cleavable Peptide.



Supporting Information 2. Number Mean Curve of the Three groups at 24 h.



Supporting Information 3. Assessment of MMP-7 Responsiveness at Different Concentrations of Nanocarriers Through the Change of Hydrodynamic Radii Over Time.

	Number Mean (nm)					
MMP-7 Concentration	**50 nM**		**1 nM**		**control**	
	Mean	SD	Mean	SD	Mean	SD
0 h	276.633	6.716	276.633	6.716	276.633	6.716
2 h	229.820	25.122	241.913	53.115	249.989	28.346
4 h	241.267	50.813	246.611	15.461	252.889	20.693
6 h	247.300	42.757	210.978	36.231	235.700	28.838
10 h	287.450	24.253	202.129	38.369	211.813	47.141
24 h	0.8725	0.2864	178.390	35.651	199.029	29.836