# Chapter 15

# Enzyme-Responsive Nanoparticles for the Treatment of Disease

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## Abstract

Nanomedicine for cancer therapy seeks to treat malignancies through the selective accumulation of therapeutics in diseased tissue. Nanoparticles offer the convenience of high drug loading capacities and can be readily decorated with targeting moieties, drugs, and/or diagnostics. Our lab has pioneered a new tissue targeting strategy where enhanced accumulation of nanomaterials occurs as a result of morphology changes to the material in response to overexpressed enzymes in diseased tissues. Herein, we describe the general strategy for the preparation of these enzyme-responsive nanoparticles (ER-NPs) for therapeutic applications.

**Key words** Nanoparticles, Enzyme-responsive nanoparticles, Self-assembly, Nanomedicine

## 1 Introduction

A central objective of nanomedicine is to expand the therapeutic window of small molecule drugs by packaging them in nanoscale carriers that shield them from the body until they reach their intended target, where the payload is then released. Overall, the goal is to increase the efficacy of the therapeutic, while decreasing its toxicity associated with off-target accumulation. Toward this end, vast arrays of nanoparticle systems have been developed, based on both passive accumulation methods, such as the enhanced permeability and retention (EPR) effect in tumors [1–3], and active accumulation mechanisms, such as receptor-ligand association [4–6], pH gradients [7–9], redox processes [10–12], and exogenous stimuli [13, 14].

The Gianneschi lab has developed a new type of nanoparticle system that is designed to respond to enzymatic signals endogenous to diseased tissue [15–20]. The system utilizes an active accumulation mechanism that is distinctly different from conventional methods. Instead of relying on the inherent $K_D$ of receptor-ligand binding to accumulate material at disease sites, we instead take

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advantage of the kinetics of enzymatic action and catalytic amplification to retain materials. Specifically, the materials are designed to respond to matrix metalloproteinases (MMPs), which are proteases upregulated in the progression of many inflammatory diseases, including certain cancers [21–24] and post-myocardial infarction [25–28]. Our materials, herein referred to as enzyme-responsive nanoparticles (ER-NPs), are functionalized with MMP-responsive peptides on the nanoparticle shell. When exposed to MMPs, the peptides are cleaved at their recognition sequences. This cleavage disrupts the nanoparticle structure and induces a nano- to micro-scale morphology change in the ER-NPs. For in vivo applications, this translates to long retention times (on the order of days to weeks) in the tissue of interest, making it possible to deliver therapeutic cargo [15] or imaging agents [16, 17, 20].

A key feature of our materials is that they can be prepared with a multitude of functionalities in a well-controlled manner in high fidelity. This is achieved, in part, by preparing the polymer building blocks for particle assembly via Ring Opening Metathesis Polymerization (ROMP). ROMP is a living polymerization technique [29–34], where initiators are capable of producing well-defined, highly reproducible block copolymers with exceptionally low dispersities. Because these materials are generated with ROMP, one can precisely control block lengths and can incorporate multiple functional moieties in a single system. Figure 1 shows a general scheme for how this is accomplished. With this methodology one can polymerize peptides, hydrophobic drugs, and diagnostic agents, all of which are key components of the ER-NP systems. The utility of the approach makes it easy to envision expanding this technology to include other high-value molecular cargo.

Irrespective of application, the enzyme responsive nanoparticles (ER-NPs) follow a common synthetic scheme in their formation and are programmed to respond following exposure to MMPs (Fig. 1). In general, amphiphilic block copolymers (Fig. 1a) containing the appropriate cargo (drug or diagnostic agent) and MMP-responsive peptides are generated via ROMP, which assemble into nanoparticles upon dialysis into aqueous medium from a suitable organic solvent (Fig. 1b). Once assembled, the nanoparticles are characterized via DLS and TEM and assayed for susceptibility to MMPs (Fig. 1c). Finally, the nanoparticles are investigated in their intended applications. Herein, we present a general strategy for the preparation of ER-NPs for therapeutic applications.

## 2 Materials

### 2.1 Peptides and Peptide-based ROMP Monomers

1. Fmoc-protected amino acids (L- and D-versions) for solid phase peptide synthesis (see **Note 1**).
2. Peptide synthesis resins (*see* **Note 2**).

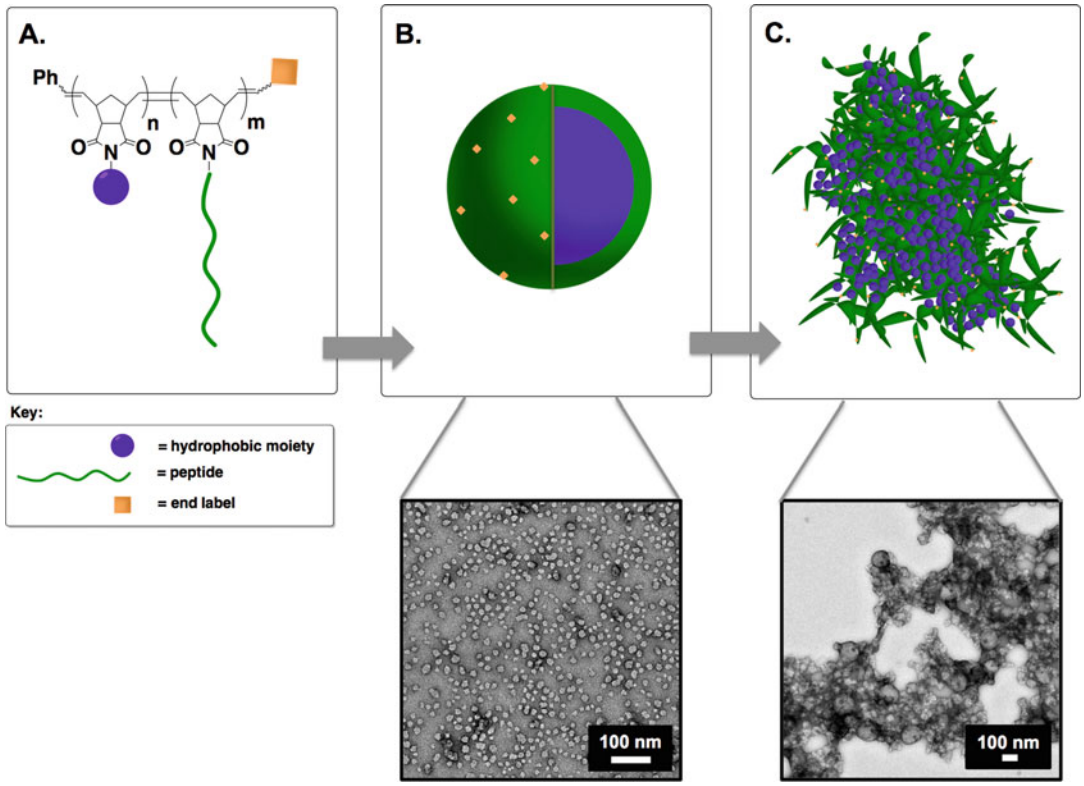


**Fig. 1** General Functional Scheme of ER-NPs. (**a**) Generalized ROMP peptide polymer amphiphile structure, where the hydrophobic moiety may be a drug or inert material, the peptide is MMP-responsive, and the end-label may be a diagnostic agent. (**b**) Upon dialysis, the peptide polymer amphiphiles assemble into nanoparticles, with the hydrophobic moiety in the core and peptide (and diagnostic agent, if used) coating the outside. Shown is a representative TEM image of ER-NPs synthesized by our lab. (c) When exposed to MMPs, the ER-NPs undergo a drastic morphology change. Shown is a representative TEM image depicting such a change. TEM images reproduced with permission from [15]

3. 20% methyl piperidine in DMF for Fmoc deprotection.
4. 0.4 M HBTU in DMF.
5. 1 M DIPEA in DMF.
6. *N*-(Glycine)-*cis*-5-norbornene-*exo*-2,3-dicarboximide [35].
7. Resin-cleavage cocktail solution (*see* **Note 2**).
8. Cold diethyl ether.

### *2.2 Ring Opening Metathesis Polymerization: Direct Method (see Note 3)*

1. Catalyst—$(IMesH_2)(C_5H_5N_2)(Cl)_2Ru{=}CHPh$ [33].
2. Peptide monomer.
3. Hydrophobic drug monomer [15].
4. Dye-labeled termination agents [36].
5. Dry DMF.

6. Ethyl vinyl ether.
7. Inert atmosphere.
8. Air-free reaction vessel.
9. Magnetic stir bar and stir plate.
10. Diethyl ether.
11. Size Exclusion Chromatography with Multi-Angle Light Scattering (SEC-MALS) for polymer analysis.

### 2.3 Ring Opening Metathesis Polymerization: Post-Polymerization Modification Method (see Note 3)

1. Catalyst—$(IMesH_2)(C_5H_5N_2)(Cl)_2Ru{=}CHPh$ [33].
2. NHS-norbornenyl ester [18].
3. Enzyme-responsive peptide (*see* **Note 1**).
4. Hydrophobic drug monomer [15].
5. Dye-based chain transfer agents [36] for dye-labeled polymer ends.
6. NHS-modified dye and amine monomer for post-polymerization incorporation of dyes [18].
7. Dry DMF.
8. Ethyl vinyl ether.
9. Inert ($N_2$) atmosphere.
10. Air-free reaction vessel (or glove box with inert atmosphere).
11. Magnetic stir bar and stir plate.
12. Diethyl ether.
13. SEC-MALS for polymer analysis.
14. Centrifuge.

### 2.4 Dialysis Reagents

1. ROMP polymer.
2. Dimethyl Sulfoxide (DMSO).
3. DMF.
4. Dulbecco's Phosphate Buffered Saline (DPBS): 2.67 mM KCl, 1.47 mM $KH_2PO_4$, 138 mM NaCl, 8.09 mM $Na_2HPO_4$, pH = 7.4 (*see* **Note 4**).
5. 3500 MWCO dialysis tubing and/or cups.
6. Dynamic Light Scattering (DLS) instrument for hydrodynamic radius determination.

### 2.5 Matrix Metalloproteinase Degradation of ER-NPs

1. ER-NPs.
2. MMP-9 (Calbiochem, USA).
3. 24 mM *p*-aminophenyl mercuric acetate in 0.1 M NaOH.
4. Heating Block.
5. RP-HPLC for analysis.

### 2.6 Transmission Electron Microscopy

1. ER-NPs.
2. TEM carbon grids (Ted Pella Inc.).
3. Emitech K350 glow discharge unit.
4. 1% uranyl acetate stain.
5. FEI Tecnai G2 Sphera Microscope.

## 3 Methods

### 3.1 Preparation of Peptide Substrates

1. Determine peptide sequence to be synthesized (*see* **Note 1** and **Note 5**).
2. If using a peptide synthesizer, program sequence into unit and initiate synthesis based on the instrument being used. In this case, skip **steps 3–8**.
3. If synthesizing by hand, weigh out the appropriate amounts of amino acids and resin, based on desired coupling conditions (*see* **Note 2**).
4. Swell the resin of choice (*see* **Note 2**) for 45 min with DMF in a peptide synthesis vessel.
5. Add first amino acid to vessel.
6. Add HBTU and DIPEA to vessel (resin/amino acid/HBTU/DIPEA 1:3:3:4) and shake for 45 min.
7. Drain vessel; add 20% 4-methylpiperidine in DMF and shake for 20 min for Fmoc deprotection.
8. Add next amino acid to vessel, repeat **steps 6–7**. Repeat until the entire sequence is synthesized.
9. If synthesizing a peptide to be used directly as a monomer for ROMP (*see* **Note 2**), couple *N*-(Glycine)-*cis*-5-norbornene-*exo*-2,3-dicarboximide [35] at the last step, using the same coupling conditions as in **steps 6–7**.
10. Cleave the peptide from the resin, using the correct cleavage cocktail for resin (*see* **Note 2**).

### 3.2 Ring Opening Metathesis Polymerization: Direct Polymerization Method (see Note 3 and Fig. 2)

1. Determine desired block lengths (*see* **Note 6**), and calculate amounts of all monomers and catalyst needed.
2. In a flask that has been flushed with $N_2$ (or in a glove box), dissolve hydrophobic drug monomer in dry DMF at a concentration between 0.1 and 0.8 M.
3. In a separate vessel, dissolve catalyst in dry DMF at a concentration between 0.01 and 0.05 M.
4. Quickly add correct volume of catalyst to hydrophobic drug monomer to initiate polymerization.

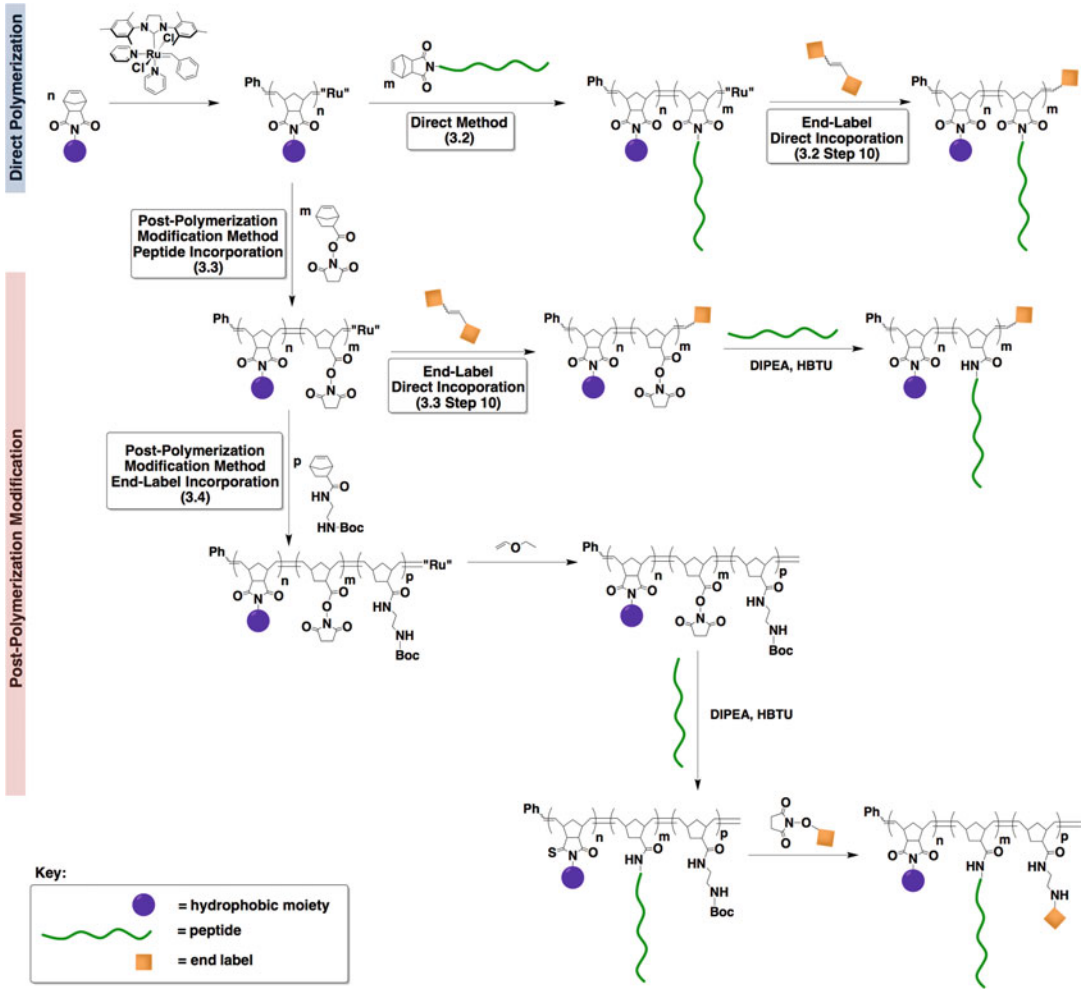


**Fig. 2 General Synthetic Scheme for Generation of Enzyme-Responsive ROMP Polymers**. The first block of our peptide polymer amphiphiles is polymerized in the same manner, regardless of peptide addition method (direct or post-polymerization modification). After the completion of the first block, however, two different directions may be taken—direct polymerization of the functional material/peptide (proceeding to the right of the first block in the scheme), or polymerization of a functionalizable norbornenyl derivative for post-polymerization modification (proceeding below the first block in the scheme)

5. Stir reaction with magnetic stir bar for 2 h to ensure complete polymerization of drug monomer.
6. Remove a 30 μL aliquot for SEC-MALS analysis of the homopolymer (the first block of the copolymer). Quench aliquot with ~1 μL ethyl vinyl ether for 20 min before analyzing (Fig. 3a).
7. Dissolve peptide monomer in dry DMF at a concentration of ~0.05 M.

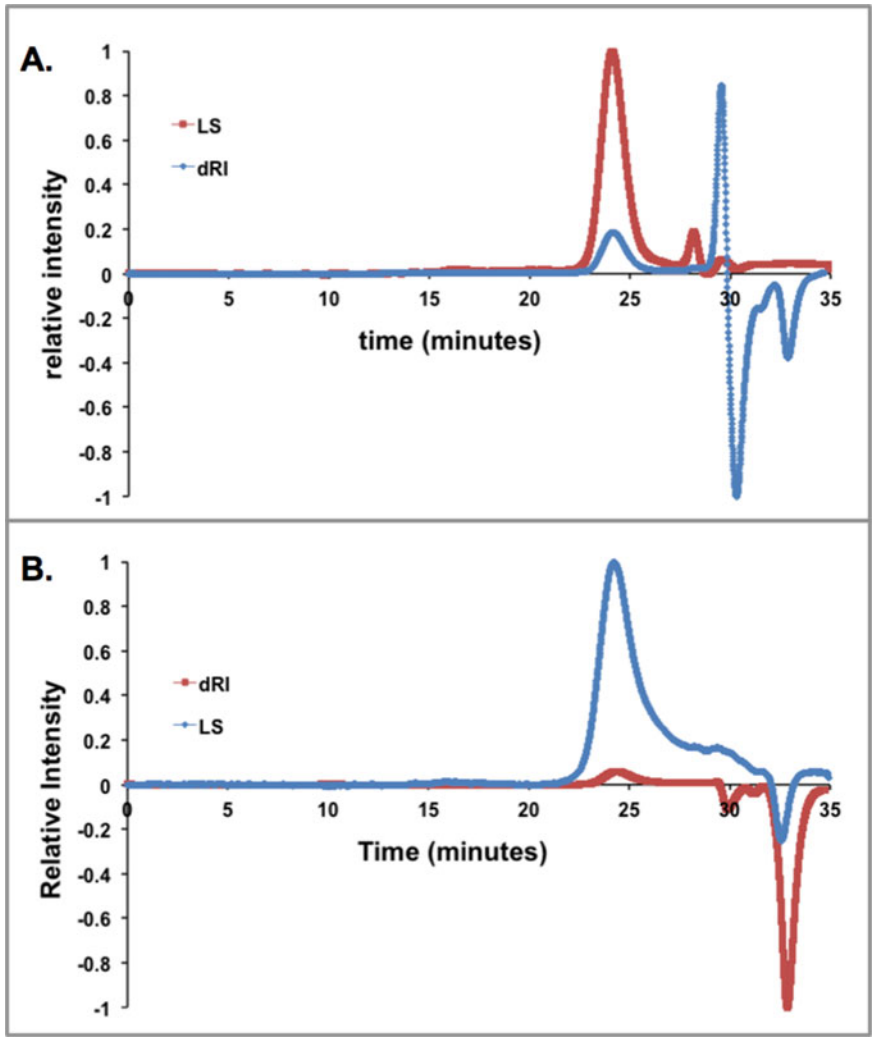


**Fig. 3** SEC-MALS Traces of ROMP polymers. To determine molecular weight, materials are analyzed via SEC-MALS after A) polymerization of the first (hydrophobic) block and B) polymerization of the second (hydrophilic) block. Polymer sequence: $(Paclitaxel)_{10}$-$(NorG\text{-}GPLGLAGGERDG)_3$. Source: Reproduced with permission from [15]

8. Add appropriate volume of peptide monomer to vessel containing the remaining solution. Stir for 3 h to ensure complete polymerization (*see* **Note** 7).
9. Remove a 30 μL aliquot for SEC-MALS analysis of the diblock copolymer. Quench aliquot with ~1 μL ethyl vinyl ether for 20 min before analyzing (Fig. 3b).
10. If incorporating a dye as an end-label, add 1.2 equivalents of the desired dye-based chain transfer agent and stir for an additional 2 h.

11. Quench catalyst by addition of ethyl vinyl ether; stir 25 min to ensure complete quenching.
12. Transfer polymer solution to a 50 mL centrifuge tube and reduce DMF volume to <2 mL.
13. Precipitate polymer from solution by adding ~40 mL of cold diethyl ether to centrifuge tube. Centrifuge at 4000 rpm in a microcentrifuge for 7 min. Decant ether, and dry polymer cake *in vacuo* overnight.

### 3.3 Ring Opening Metathesis Polymerization: Post-Polymerization Modification for Peptide Incorporation

1. Determine desired block lengths (*see* **Note 6**), and calculate amounts of all monomers and catalyst needed.
2. In a flask that has been flushed with $N_2$ (or, in a glove box), dissolve hydrophobic drug monomer in dry DMF at a concentration between 0.1 and 0.8 M.
3. In a separate vessel, dissolve catalyst in dry DMF at a concentration between 0.01 and 0.05 M.
4. Quickly add correct volume of catalyst to hydrophobic drug monomer to initiate polymerization.
5. Stir reaction with magnetic stir bar for 2 h to ensure complete polymerization of drug monomer.
6. Remove a 30 μL aliquot for SEC-MALS analysis of the homopolymer (the first block of the copolymer). Quench aliquot with ~1 μL ethyl vinyl ether for 20 min before analyzing.
7. Dissolve NHS-norbornenyl ester in dry DMF at a concentration of 0.1–0.7 M.
8. Add appropriate volume of NHS-norbornenyl ester to vessel containing the remaining solution. Stir for 40 min to ensure complete polymerization.
9. Remove a 30 μL aliquot for SEC-MALS analysis of the diblock copolymer. Quench aliquot with ~1 μL ethyl vinyl ether for 20 min before analyzing.
10. If incorporating a dye as an end-label, add 1.2 equivalents of the desired dye-based chain transfer agent and stir for an additional 2 h.
11. Quench catalyst by addition of ethyl vinyl ether; stir 25 min to ensure complete quenching.
12. Transfer polymer solution to a 50 mL centrifuge tube and reduce DMF volume to <2 mL.
13. Precipitate polymer from solution by adding ~40 mL of cold diethyl ether to centrifuge tube. Centrifuge at 4000 rpm for 7 min. Decant ether, and dry polymer cake *in vacuo* overnight.
14. Dissolve dried polymer in 4:1 DMF:DMSO.

15. Add desired number of equivalents of peptide (*see* **Note 6**) to flask.
16. Add excess (4–16 equivalents) DIPEA to flask.
17. Stir at room temperature overnight.
18. Precipitate polymer with cold diethyl ether. Centrifuge.
19. Decant ether and wash precipitate with cold methanol twice.
20. Dry newly synthesized peptide-polymer amphiphile (PPA) *in vacuo* overnight.
21. Analyze degree of peptide conjugation by SEC-MALS against unconjugated polymer.

### *3.4 Ring Opening Metathesis Polymerization: Post-Polymerization Modification for Dye Incorporation*

1. If following direct polymerization method, follow **steps 1–9** in Subheading 3.2. If following post-polymerization modification method, follow **steps 1–9** in Subheading 3.3.
2. Dissolve amine monomer in dry DMF at a concentration of ~0.06 M.
3. Add appropriate volume of amine monomer to vessel containing the remaining solution. Stir for 40 min to ensure complete polymerization.
4. Remove a 30 μL aliquot for SEC-MALS analysis of the diblock copolymer. Quench aliquot with ~1 μL ethyl vinyl ether for 20 min before analyzing.
5. Quench catalyst by addition of ethyl vinyl ether; stir 25 min to ensure complete quenching.
6. Transfer polymer solution to a 50 mL centrifuge tube and reduce DMF volume to <2 mL.
7. Precipitate polymer from solution by adding ~40 mL of cold diethyl ether to centrifuge tube. Centrifuge at 4000 rpm for 7 min. Decant ether, and dry polymer cake *in vacuo* overnight.
8. If following direct polymerization method, skip to **step 16**.
9. If following post-polymerization modification method, dissolve dried polymer in 4:1 DMF:DMSO.
10. Add desired number of equivalents of peptide (*see* **Note 6**) to flask.
11. Add excess (4–16 equivalents) DIPEA to flask.
12. Stir at room temperature overnight.
13. Precipitate polymer with cold diethyl ether. Centrifuge at 4000 rpm in a microcentrifuge for 7 min.
14. Decant ether and wash precipitate with cold methanol twice.
15. Dry newly synthesized peptide-polymer amphiphile (PPA) *in vacuo* overnight.
16. Dissolve polymer in DMF.

17. Add 1.2 equivalents of desired NHS-modified dye to the polymer solution.
18. Add 1.2 equivalents of DIPEA to the polymer solution.
19. Stir overnight at room temperature.
20. Precipitate polymer with cold diethyl ether. Centrifuge at 4000 rpm in a microcentrifuge for 7 min.
21. Decant ether and dry polymer *in vacuo* overnight.

### 3.5 ER-NP Formation

1. Dissolve polymer in DMSO or DMF at a concentration of 0.1–10.0 mg/mL (*see* **Note 8**).
2. In a drop-wise fashion, slowly add 1.0–1.5 volume equivalents of DPBS (*see* **Note 4** and **Note 9**) to facilitate ER-NP formation.
3. Transfer the ER-NP solution to dialysis tubing (or dialysis cup) and dialyze into 1.0 L of DPBS.
4. Change DPBS twice daily for three total water changes.
5. Remove ER-NPs from dialysis tubing/cups and analyze size distributions by DLS (Fig. 4a).
6. Store in vials at 4 °C.

### 3.6 Analysis of ER-NPs by TEM (Fig. 4b)

1. Glow discharge and plasma-clean carbon grid.
2. Place small (4 μL) aliquot of ER-NP on grid. Allow to adhere for 5 min.
3. Rinse grid with distilled water.
4. Add 1% uranyl acetate solution (stain) to grid. Rinse immediately with water.
5. Allow sample to dry for 5 min.
6. Analyze with a microscope.

### 3.7 Enzymatic Degradation of ER-NPs with MMPs

1. Activate MMP-9 by adding 0.4 μL of a 24 mM *p*-aminophenyl mercuric acetate in 0.1 M NaOH to 5 μL enzyme solution, and heating for 2 h at 37 °C (*see* **Note 10**).
2. In one vial, treat 100–120 μM ER-NP (concentration with respect to peptide) with MMP-9 (100 μU, 1.25 μL) for 24 h at 37 °C.
3. As a positive control, treat 100–120 μM of authentic peptide with MMP-9 (100 μU, 1.25 μL) for 24 h at 37 °C.
4. As a negative control, heat 100–120 μM of authentic peptide for 24 h at 37 °C.
5. Inactivate MMP-9 by heating solutions to 65 °C for 20 min.
6. Analyze all samples with RP-HPLC, collecting the peaks corresponding to peptide fragments for ESI analysis.

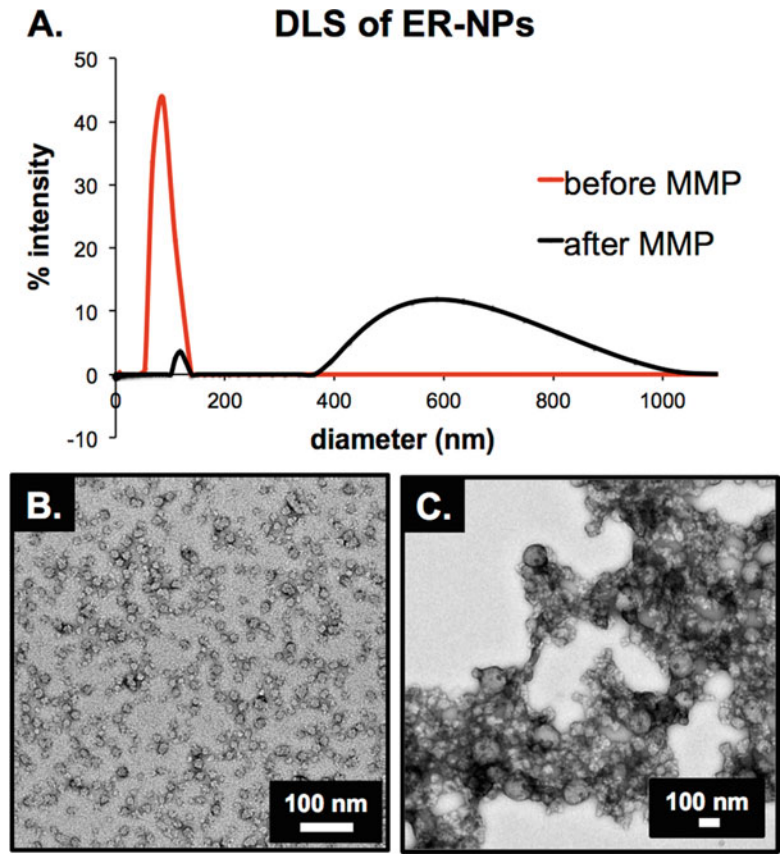


**Fig. 4** Characterization of ER-NPs. (**a**) DLS trace of ER-NPs before treatment with MMP (red trace) and after treatment with MMP (black trace). (**b**) Negative stain, dry-state TEM image of ER-NPs before treatment with MMP. (**c**) Negative stain, dry-state TEM image of ER-NPs after exposure to MMP. Polymer sequence: $(\text{Paclitaxel})_{10}$-$(\text{NorG-GPLGLAGGERDG})_3$. Source: Reproduced with permission from [15]

The cleavage efficiency can be calculated from the fraction of the area under the peak, relative to the area of the negative control (Fig. 5).

7. Analyze aggregation of ER-NPs by DLS and TEM (Fig. 4b, c).

## 4 Notes

1. In our experience, the peptide sequence to be used for forming ER-NPs does not have to be exactly the same in every application—however, all peptide sequences we use contain the recognition sequence **GPLGLAG**. MMP-9 and -12 cleave

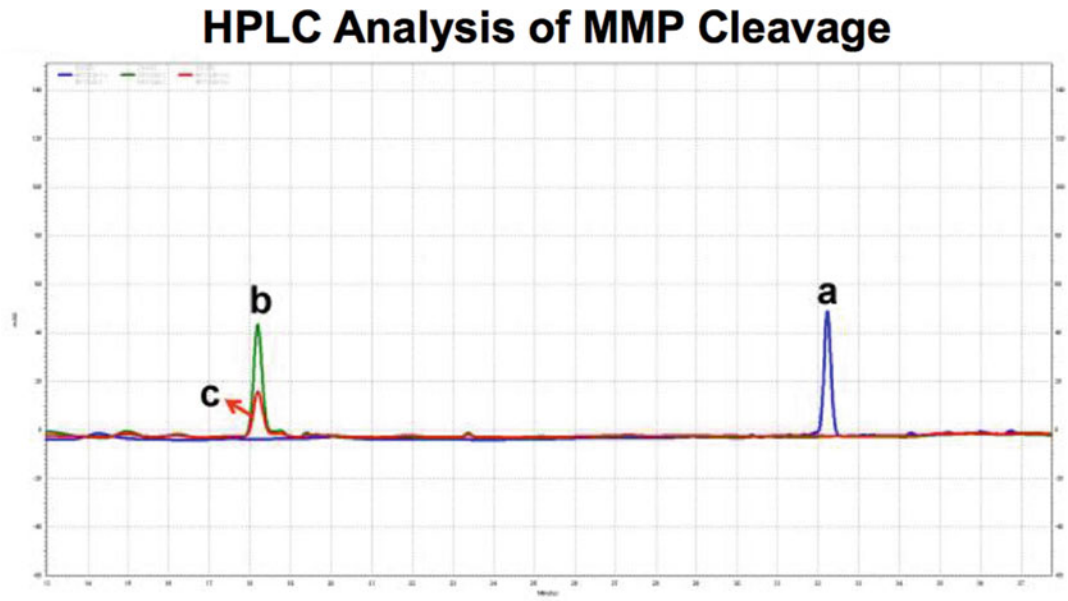


**Fig. 5** HPLC Analysis of ER-NPs for MMP cleavage efficiency. Peak "a" corresponds to an authentic sample of the intact peptide, prior to MMP degradation. Peak "b" corresponds to the degradation product of the authentic peptide sample, after exposure to MMP. Peak "c" corresponds to the degradation product of the peptide on the ER-NP, after exposure to MMP. Efficiency of peptide cleavage on the ER-NP by MMP is calculated from the ratio of the area under peak "c" to that of peak "b." Polymer sequence: $(Phenyl)_{21}$-[(Nor-$COOH)_3$ -(Nor-$GPLGLAGGERDG)_3$], where "Nor" represents norbornenyl derivative. Source: Reproduced with permission from [17]

this sequence at the residues underlined. Additional amino acids on the C-terminal end of the recognition sequence may be added, based on the needs of the researcher. Things to consider on this front include the overall charge of the peptide, the hydrophilicity of the peptide, and whether or not the peptide will be directly incorporated into a ROMP monomer [37]. The sequences that we have had success with in our lab include:

GPLGLAGGWGERDGS [16, 17, 20].
GPLGLAGGERDG [15, 19].
GPLGLAGKWAAAAKAAAAK [18]

2. The choice of resin for solid phase peptide synthesis (SPPS) will depend upon the peptide sequence to be made. In our publications, we use several different resins (Rink Amide, MBHA, and Wang). Thus, the cleavage cocktails used will also depend on the choice of resin and amino acid sequence, the protocols for which can be readily found in the literature.
3. One of the benefits of ER-NPs is that their substituent ROMP polymers can be generated in different ways, yet still have the same resulting function as nanoparticles. Indeed, our lab has

successfully utilized several different polymerization schemes toward the formation of ER-NPs, which suggests that this technique is robust. Figure 1 outlines the polymerization routes we have utilized, which should serve as a useful guide in determining which synthetic route the researcher should utilize.

4. When dialyzing from organic solvent to aqueous medium, it may be necessary to use a different buffer than what is indicated in this text. We have found that, when working with certain peptide sequences, PBS solutions that contain calcium and magnesium lead to aggregation of polymers, rather than nanoparticle formation. Alternative buffers that have worked for our group include pure deionized water; Tris(hydroxymethyl)aminomethane (Tris) buffer: 50 mM, pH=8.5; and phosphate buffered saline (PBS): 40 mM $Na_2PO_4$, pH=8.0.
5. As a negative control for ER-NP function, we synthesize D-amino acid versions (D-peptides) of all peptides and generate polymers with them. To do this, split the polymer solutions in half before adding the second block in the direct polymerization method, and add L-peptide monomers to one portion and D-peptide monomers to the second.
6. In our work, we have successfully generated ER-NPs with hydrophobic:hydrophilic ratios of 21:8 [17, 18], 10:3 [15, 19], and 20:2 [20]. However, the actual lengths of each block (hydrophobic and hydrophilic) may need to be adjusted by the researcher to generate well-defined nanoparticles. If using the direct polymerization method for the peptide component, it is important to note that the degree of polymerization of the peptide will impact its susceptibility to proteolysis by MMPs [38], with longer block lengths showing marked decrease in susceptibility.
7. The time required for complete polymerization of the peptide block will depend upon concentration and sequence identity [37]. Therefore, when working with a new peptide monomer, it is best to monitor the polymerization by NMR to determine the length of time needed to fully polymerize the peptide. This is achieved by monitoring the disappearance of the monomer's olefin peak at ~6.30 ppm and the appearance of the polymer's cis/trans olefin peaks at 5.73 and 5.50 ppm (Fig. 6).
8. It may be necessary to use a different organic solvent for dissolving the polymer, depending on the polymer components. DMSO and DMF work well in general, but other solvents that are miscible with water may be needed to form well-defined spherical nanoparticles. In addition, the initial polymer concentration may need to be adjusted so that aggregation does not occur. It is therefore recommended to complete a small solvent and concentration screen, to determine the ideal parameters for each specific system.

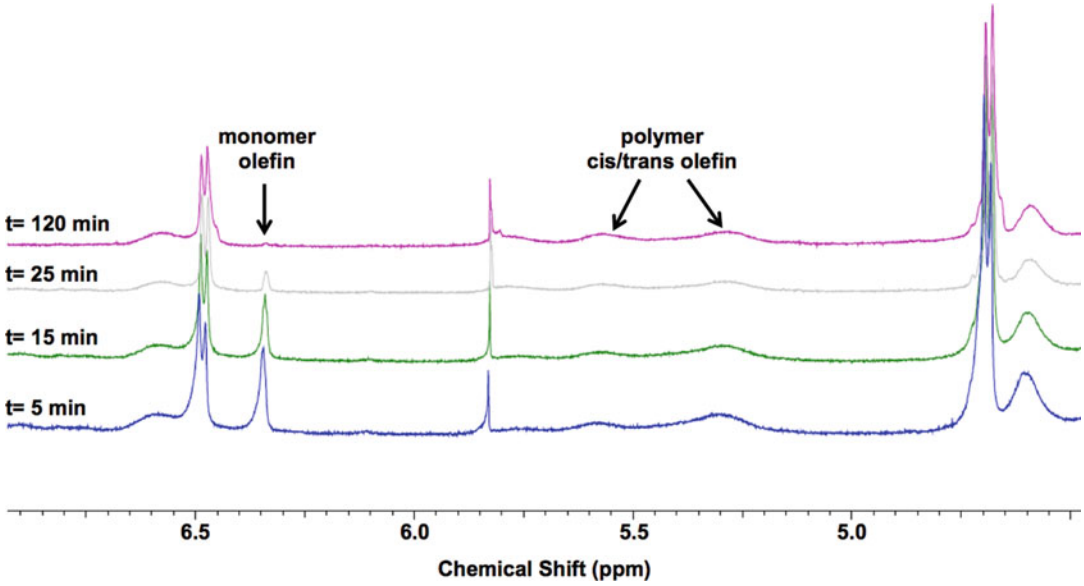


**Fig. 6** Monitoring Polymerization by $^{1}$H–NMR. It is possible to monitor polymerization progression by NMR by observing the disappearance of the monomer olefin peak (~6.30 ppm) and appearance of the polymer cis/trans olefin peaks (~5.7 and ~5.2 ppm) as a function of time. In this representative example, some of the monomer is already consumed at t=5 min (blue trace), as evidenced by the appearance of the cis/trans peaks. The polymerization proceeds until t=120 min (magenta trace), when the monomer olefin peak has completely disappeared

9. Slow addition of aqueous buffer to the polymer solution is important—generally, a slower addition leads to better-defined nanomaterials and reduces the chances of unwanted aggregation. It may be useful to use a syringe pump to control the addition of buffer [20], and extra care should be taken until the critical water concentration (~30–50% by volume) is reached. This parameter, as with the choice of buffers, may need to be adjusted based on the specific polymer system.
10. Though this protocol uses MMP-9, we have also had success with MMP-2 and MMP-12.

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