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Polymer Capsules for Plaque-Targeted *In Vivo* Delivery

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Polymer capsules are currently being developed for biomedical applications ranging from sensing and confined bio-reactions to drug delivery.[1] These applications require polymer capsules to be loaded with functional cargo, such as liposomes, nanoparticles, enzymes, or therapeutics, and targeted to the specific site of action, such as plaques, tumors, or organs.[2] The functional cargo is required to be preferentially retained in the capsules either by using cargo-impermeable capsules, or by conjugating the cargo to the polymer shell.[3] Conjugation offers some advantages, such as chemical control over the linking moieties, which can be pH, redox or enzyme responsive, while cargo-impermeable capsules offer advantages such as high drug loading and responsiveness based on the polymer shell properties, for example enzymatic degradation, and pH- or salt-induced swelling.[4] Besides the retention of functional cargo, polymer capsules for biomedical application also require stealth and targeting

functionalities for site-specific drug delivery.[5] By controlling the surface chemistry of the capsules, their interaction with the surrounding environment may be modulated, and therefore specific functions that arise from the encapsulated cargo can be localized to specific environments that are targeted by the capsules.[6] Therefore, cargo retention and localization are two necessary requirements for effective *in vivo* drug delivery. Herein, by building on a polymer capsule platform with high drug loading and small-molecule retention that we recently introduced,[2a] we demonstrate *in vivo* targeted delivery of therapeutic-loaded capsules to unstable atherosclerotic plaques, the main cause of myocardial infarction and stroke.[7]

Current options for plaque treatment are either highly invasive (catheter balloon and stenting) or require life-long, high-dose medication resulting in considerable side effects. Ideally, therapeutic delivery to plaques must be specific to avoid off target effects to healthy tissue.[8] In addition, plaque development and progression is an ongoing process so a slow release of drugs over a long period of time is required, (e.g., several weeks vs a few days). Current therapeutic options such as systemic statin treatment provides an effective means to moderate the cardiovascular risks of hyperlipidemia and shows promising results in secondary prevention of cardiovascular disease but fail to prevent the rupture of highly inflamed plaques responsible for acute thrombotic events.[9] Nanomedicine with targeted, responsive carriers has the potential to close this therapeutic gap.[10] Recently, our group introduced the use of polymer-stabilized calcium carbonate ($CaCO_3$) particles for the rapid, versatile and efficient preparation of loaded polymer capsules (**Figure 1**).[2a] Monodisperse polymer-stabilized $CaCO_3$ particles can be formed simply by varying the reaction time, with shorter reaction times leading to smaller particles.[2a] After synthesis these porous, polymer-impregnated particles allow for a high degree of drug loading and drug retention due to the aromatic groups of the stabilizing polymer, charge association from both the calcium carbonate and the

stabilizing polymer, capillary effects from the pores, etc. Importantly, no complicated engineering or chemistries are required for loading, as can be the case for some other polymer capsule-based systems.[2a] Herein, we use polymer-stabilized $CaCO_3$ particles to prepare drug loaded sub-micrometer degradable polymer capsules with stealth and targeting moieties. To prepare these capsules, poly(styrenesulfonate) (PSS)-stabilized $CaCO_3$ particles (**Figure 1B**) were loaded with a therapeutic matrix metalloprotease blocking fluorophore (naphthofluorescein) and capped with a trifunctional polymer. The naphthofluorescein loses its fluorescence upon encapsulation, making it potentially suitable for self-reporting successful delivery and release. The trifunctional polymer was composed of a pH sensitive component (poly(2-diisopropylaminoethyl methacrylate), PDPA), a stealth component (poly(ethylene glycol), PEG), and a single azide click moiety (from the polymer initiator) (**Figure 1C-E and Figure S1**).[11] PDPA was chosen due to its physiologically-relevant protonation at pH ~6.5 that allows for a pH dependent release of drugs,[12] while PEG was chosen to reduce the fouling and non-specific binding of the capsules. The polymer was a random co-polymer with a PDPA to PEG ratio of 5:2, as determined by NMR (**Figure S1**). The PDPA capsules were stable in water after $CaCO_3$ removal with ethylenediaminetetraacetic acid (EDTA) and retained the template size of ~700 nm (**Figure S2)**. However, the PDPA capsules were not stable in human plasma and released the cargo within 5 min (**Figure 2**). We found that plasma filtered through a ~10 kDa cut-off filter, to only obtain small molecules such as fats, hormones, and small peptides, did not lead to the immediate release of naphthofluorescein, and moreover, at 4 ˚C the release was significantly hindered until the plasma was fully warmed to 37 ˚C (**Figure S3)**. Both of these observations suggest an enzymatic basis for release. However, protease inhibition, specific monoglyceride lipase inhibition, and competitive glucose and glycerol incubations did not prevent the rapid release (**Figure S4**), suggesting that proteases, specific lipases, and enzymes interacting with simple sugars were not responsible for the release. Therefore, other enzymes were likely

degrading the synthetic polymer, such as triglyceride lipases and esterases, as seen in other studies.[13] This degradation was likely because the DPA moieties are connected to the polymer backbone through ester bonds that resemble the chemical structures found in triglycerides. To identify the protein causing degradation, fast liquid protein chromatography was conducted, and PDPA capsules were incubated with each fraction collected during chromatography (**Figure S5**). Only one specific fraction, fraction 22 (F22), of the collected proteins resulted in naphthofluorescein drug release, and protein separation using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed that F22 contained a large amount of protein in the molecular weight range of 50-70 kDa. This range is where triglyceride lipases are found,[14] again suggesting a complex lipase as the main mechanism behind naphthofluorescein release.

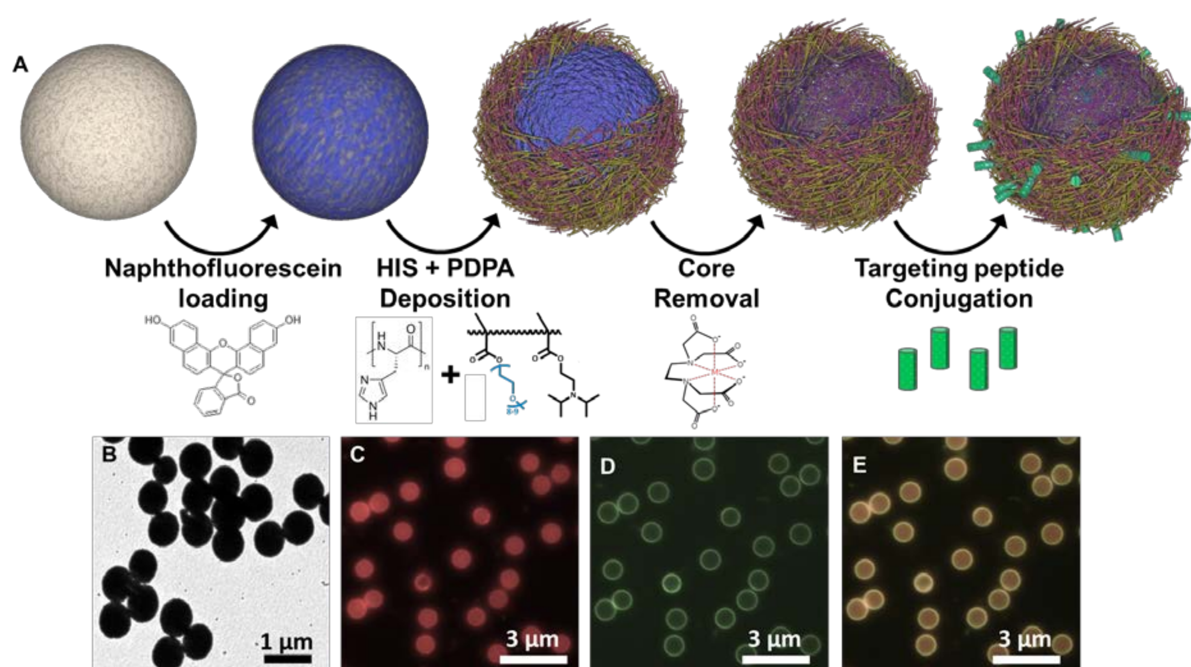

Figure 1. A) Scheme of drug loading and polymer deposition for capsule assembly, followed by targeting peptide click-conjugation. B) TEM of PSS-$CaCO_3$ template particles. C-E) Fluorescence microscopy image of rhodamine isothiocyanate (RITC) loaded (C) fluorescein isothiocyanate (FITC) labeled PDPA-HIS capsules (D) and their overlay (E).

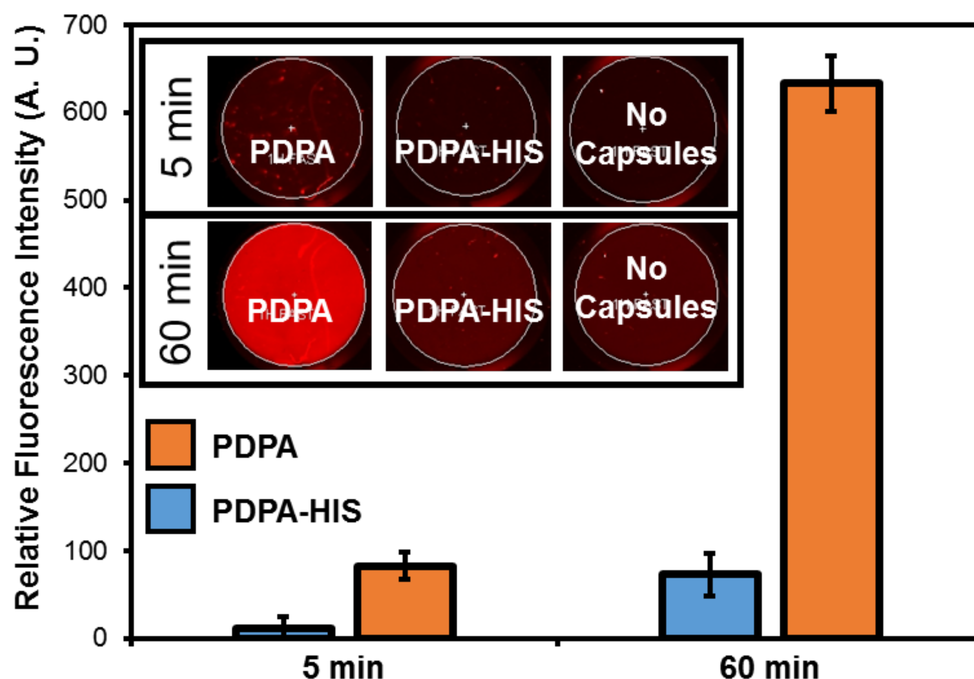

Figure 2. Comparison of PDPA and PDPA-HIS capsule stability in human plasma. Relative fluorescence intensity was calculated by subtracting the raw PDPA and PDPA-HIS values from the values generated from the control wells with no capsules. Inset, images from Odyssey scan showing little degradation for PDPA-HIS capsules, for 5 min and 60 min $p<0.01$ using ANOVA.

The burst release of the PDPA capsules upon exposure to human serum disqualified their use *in vivo*, as the capsules would not have time to reach a targeted active site before releasing the drug, and also would not qualify them for the slow release required for vulnerable plaque stabilization. Therefore, a co-stabilizing, positively charged bio-polymer, poly-L-histidine (HIS), which has recently been used to stabilize PDPA-based particles,[15] was used. HIS has nearly the same pK_a (~6.5) as PDPA and is expected to degrade *in vivo* due to enzymatic conditions. The PDPA-HIS capsules were significantly more stable in plasma than PDPA capsules (**Figure 2**). Although the PDPA-HIS capsules are composed of pH-responsive polymers, the capsules did not release the cargo under acidic environments over a long period of time (**Figure S6**), demonstrating that the capsules would need to be enzymatically degraded by non-specific proteases after internalization to release the cargo. Incubation with cell media (FCS supplemented and serum free) and macrophages did not result in significant release from the PDPA-HIS capsules.

Finally, multifunctional capsules with targeting and endosomal escape properties were engineered by copper click-functionalization of the capsules with a targeting peptide directed against MMP2 degraded collagen IV typically found in unstable atherosclerotic plaques (T-peptide, TLTYTWS).[16] The conjugation efficiency was ~82%, as measured by a DirectDetect® Spectrometer. The naphthofluoroscein loaded capsules that were functionalized with an activated cell penetrating peptide (ACPP) with an MMP2 substrate peptide linker could enter HT1080 fibrosarcoma cells, which are natural MMP2 producers (**Figure 3**). The MMP2 mediated cleavage of the ACPP releases the polycationic arm that carries the capsules and brings the capsules into the fibrosarcoma cells. The successful cellular uptake of the capsules *in vitro* prompted us to investigate the *in vivo* targeting of the targeted PDPA-HIS capsules after intravenous injection. Fluorescent, targeted PDPA-HIS capsules and control PDPA-HIS capsules with a randomized sequence (GLGYGWS) were injected into ApoE knock-out mice on a high fat diet in order to develop unstable atherosclerotic plaques after tandem stenosis surgery.[17] Unstable plaque areas were sectioned and imaged (**Figure 4**). It was apparent that the targeted capsules could selectively bind to the plaque, which requires successful evasion of the immune system and renal filtration system due to the significant proportion of PEG in the capsule shell and the fact that the capsules are larger than the renal fenestrations of 70 nm.[18] The T-peptide functionalized capsules could accumulate specifically at sites with an abundance of MMP2 degraded collagen IV, such as plaques. Single pixel analysis was used to determine the percentage of green containing pixels inside the plaque, revealing that 65% of the plaque contained fluorescence from the targeted capsules in comparison to under 5% for the control capsules (Figure 4A, C). The increased accumulation of the capsules in the liver compared to the kidneys suggests higher MMP2 activity in the liver that could present more binding sites for the T-peptide (**Figure 4, B,C and E,F**). ApoE deficient mice on high fat diets often develop fatty livers, which have greater

populations of activated Kupffer cells.[19] These specialized macrophages produce MMP2 and create more binding sites of collagen IV in the liver. Another possible reason is that the MMP2 mediated cleavage of the ACPP attached to the capsules promotes entry of the capsule into hepatocytes The kidneys show less accumulation of the capsules likely because of the renal fenestrae size threshold previously mentioned (**Figure 4C, F**).

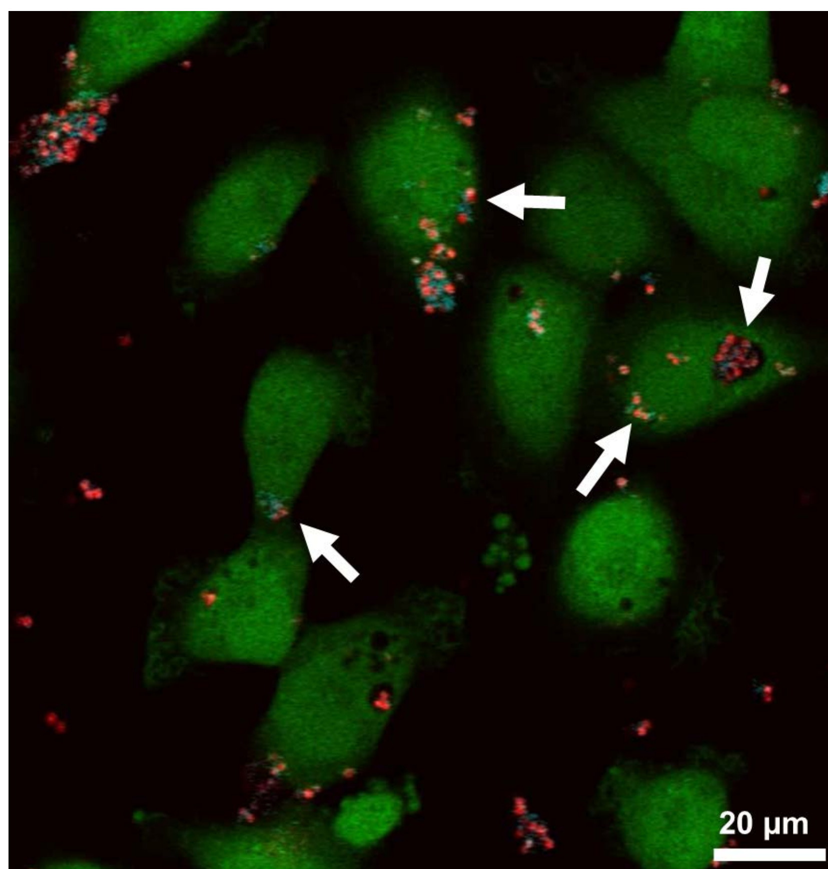

Figure 3. *In vitro* targeting of T-peptide-conjugated PDPA-HIS capsules. Confocal image Z-stack showing naphthofluorescein loaded (blue), RITC labeled (red) T-peptide-conjugated PDPA-HIS capsules targeted to HT1080 cells (green). The white arrows point to internalized and released naphthofluorescein.

In conclusion, we demonstrated that polymer capsules composed of synthetic polymers, namely PDPA, could be actively degraded in human plasma by enzymes. The synthetic PDPA capsules were stabilized with a bio-polymer (HIS), and the hybrid PDPA-HIS capsules were more stable in human plasma than PDPA alone but could still eventually degrade, which is crucial for biomedical application. Finally, the capsules were functionalized with a targeting

peptide and *in vitro* and *in vivo* targeting of the naphthofluorescein-loaded capsules was demonstrated.

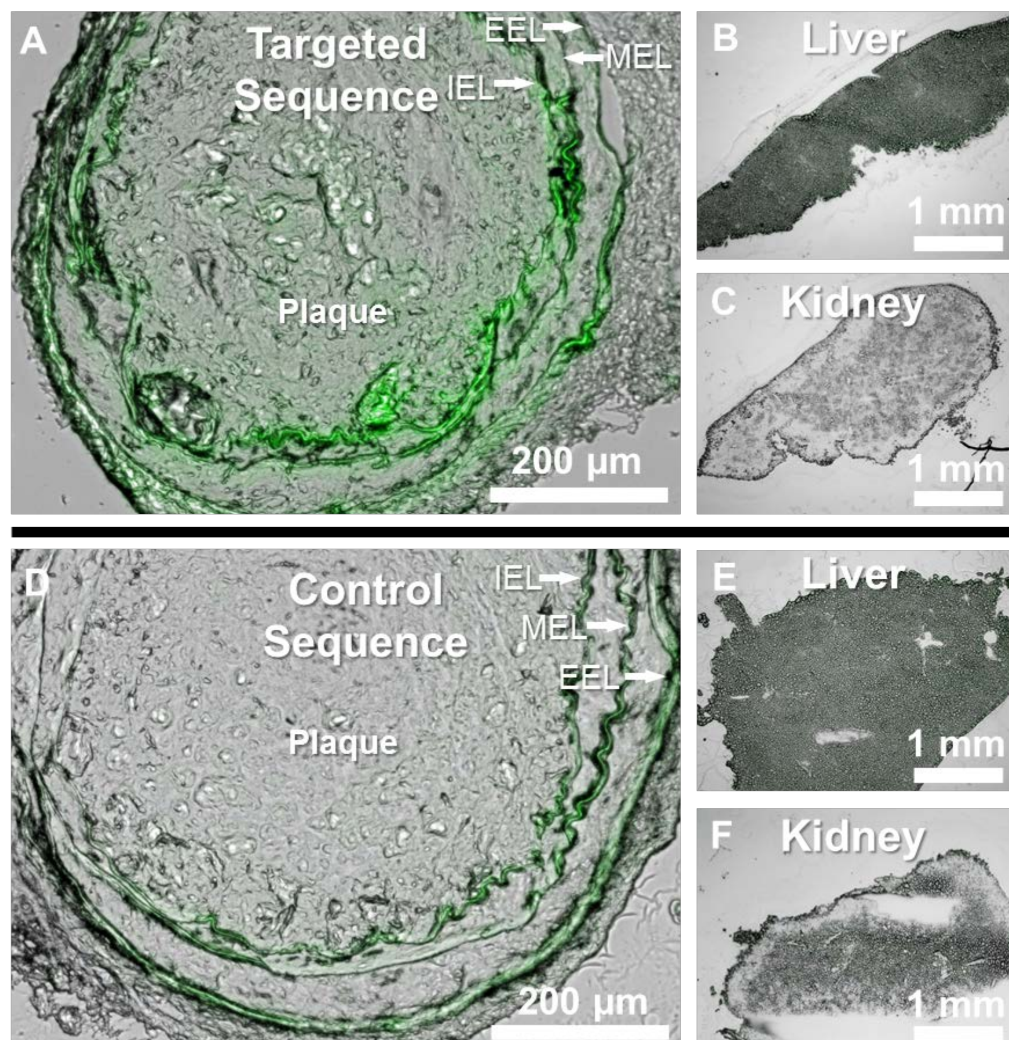

Figure 4. *In vivo* uptake of FITC labeled T-peptide-conjugated PDPA-HIS capsules (A-C) and control PDPA-HIS (D-F) into A and D, arterial unstable mouse plaque, B and E, liver, and C and F, kidney. IEL: internal elastic lamina, MEL: medial elastic lamina, EEL: external elastic lamina. Note there is strong autofluorescence of the elastic laminae of the artery in the FITC channel.

Experimental Section

Synthesis of polymer-stabilized $CaCO_3$ particles: Submicron PSS-$CaCO_3$ particles and $CaCO_3$ particles stabilized with other polymers were obtained by the fast precipitation reaction between $Ca(NO_3)_2$ and Na_2CO_3, similar to previous reports.[2a, 20] 20 mM solutions of both $Ca(NO_3)_2$ and Na_2CO_3 were separately prepared in the presence of 1 mg mL^{-1} of stabilizing polymer. 4 mL of the stabilizing polymer-Na_2CO_3 solution was quickly injected into 20 mL of

the stabilizing polymer-$Ca(NO_3)_2$ under constant stirring and the reaction was allowed to proceed for 15 min, after which the particles were spun down at 2000 *g* (1 min) and washed three times with 20 mL of ultrapure water. 15 min incubation allows for the ~700 nm particles to be formed, with shorter time periods yielding smaller particles and longer periods yielding larger particles.[2a] To wash the particles, all but 2 mL of solution was removed from the supernatant at each step, the solution pellet was then mixed vigorously, and finally 20 mL of ultrapure water was added to the tube followed by additional mixing.

Fabrication of polymer capsules: For PDPA-HIS capsules, 4 mL of HIS (1 mg mL^{-1}) dissolved in pH 5.5 (2-(N-morpholino)ethanesulfonic acid) (MES) buffer was added to the naphthofluorescein-loaded PSS-$CaCO_3$ particle suspension and incubated for 1 min. The sample was then washed 3 times in Milli-Q water. Then 6 mL of PDPA (1 mg mL^{-1}) dissolved in pH 5.5 MES buffer was added into the particle/HIS suspension and incubated for 1 min. The pH was raised by adding PBS, and the particles were washed 3 times with Milli-Q water, with a final wash step into PBS. For the PDPA capsules a similar protocol only using PDPA was followed. The $CaCO_3$ core was removed by adding 5 mL of 10 mM EDTA, followed by washing in 10 mM EDTA. Finally, the capsules were washed into PBS.

Plaque targeting experiments: Unstable plaques were induced as previously described.[17] Mice were anaesthetized by intraperitoneal injection of a ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) mixture. 200 μL of the T-peptide-conjugated PDPA-HIS capsules or random sequence-conjugated control PDPA-HIS capsules were injected via the tail vein. At the end of administration, the mice were kept anesthetized for another 30 min before the right common carotid artery segment 1 (unstable plaque) was dissected out and embedded in OCT cryomatrix for freezing. Cryosections of the frozen plaque tissue of 6 μm thickness were made using the Leica Cryostat (CM 1950, Leica Biosystems Nussloch) and mounted on glass slides. The frozen section slides were thawed for 30 min at room temperature before

examination under the 20× objective of a fluorescence microscope (IX81 Olympus microscope). Similarly, sections of the kidney and liver were imaged under the 4× objective.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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We demonstrate that targeted-polymer capsules can selectively bind to unstable plaques in mice after intravenous injection. Different formulations of the capsules are explored with a synthetic/biopolymer hybrid capsule showing the best stability and small-molecule drug retention. The synthetic polymer was comprised of pH sensitive blocks (PDPA), low binding blocks (PEG) and click-groups for post functionalization with targeting peptides specific to plaques.

Keywords: degradation, polymer blends, click-chemistry, nanomedicine, templating

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Peptide-Stabilized pH-Sensitive Polymer Capsules for Targeted *In Vivo* Drug Delivery

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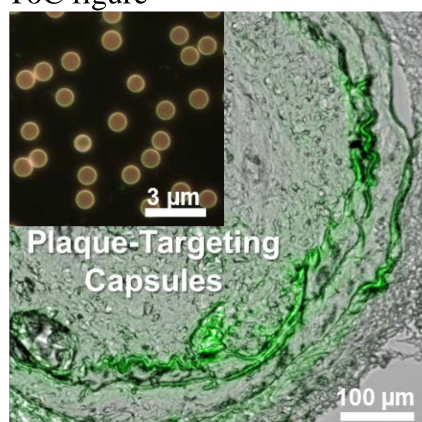

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