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# Collagen-binding nanoparticles for extracellular anti-inflammatory peptide delivery decrease platelet activation, promote endothelial migration, and suppress inflammation

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ABSTRACT

Peripheral artery disease is an atherosclerotic stenosis in the peripheral vasculature that is typically treated via percutaneous transluminal angioplasty. Deployment of the angioplasty balloon damages the endothelial layer, exposing the underlying collagen and allowing for the binding and activation of circulating platelets which initiate an inflammatory cascade leading to eventual restenosis. Here, we report on collagen-binding sulfated poly(N-isopropylacrylamide) nanoparticles that are able to target to the denuded endothelium. Once bound, these nanoparticles present a barrier that reduces cellular and platelet adhesion to the collagenous surface by 67% in whole blood and 59% in platelet-rich plasma under biologically relevant shear rates. *In vitro* studies indicate that the collagen-binding nanoparticles are able to load and release therapeutic quantities of anti-inflammatory peptides, with the particles reducing inflammation in endothelial and smooth muscle cells by 30% and 40% respectively. Once bound to collagen, the nanoparticles increased endothelial migration while avoiding uptake by smooth muscle cells, indicating that they may promote regeneration of the damaged endothelium while remaining anchored to the collagenous matrix and locally releasing anti-inflammatory peptides into the injured area. Combined, these collagen-binding nanoparticles have the potential to reduce inflammation, and the subsequent restenosis, while simultaneously promoting endothelial regeneration following balloon angioplasty.

**Statement of Significance**

In this manuscript, we present our work on the development and characterization of a novel temperature sensitive collagen-binding nanoparticle system. We demonstrate that when bound to a collagenous matrix, the nanoparticles are able to promote endothelial migration while avoiding cellular uptake. We also show that the nanoparticles are able to reduce inflammation via the release of anti-inflammatory peptides which, when combined with its ability to inhibit platelet binding, could lead to reduced intimal hyperplasia following balloon angioplasty. The drug delivery platform presented represents a unique dual therapy biomaterial wherein the nanoparticle itself plays a crucial role in the system's overall therapeutic potential while simultaneously releasing anti-inflammatory peptides.



## 1. Introduction

Peripheral artery disease (PAD) is characterized by an atherosclerotic stenosis within the peripheral vasculature. Despite improved screening effectiveness, PAD still affects over 25% of adults over 55, and is even more prevalent in patients with cardiovascular risk factors such as smoking and hypertension [1–6]. Additionally, PAD is associated with increased risk of other cardiovascular diseases, making the development of an effective long-term treatment of paramount importance to the large population that lives with this disease [7,8].

The current standard of treatment for severe PAD is percutaneous transluminal angioplasty, sometimes coupled with stent deployment to hold open the widened vessel. A major drawback of angioplasty is that balloon deployment damages the vessel wall, thus exposing the underlying collagenous matrix [9]. Circulating platelets can then bind to the sub-endothelial collagen and become

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activated, releasing a host of pro-inflammatory factors including epidermal growth factor (EGF), IL-1β, and platelet derived growth factor (PDGF) [10–12]. Smooth muscle cells (SMC) exposed to these factors then become activated and proliferative, resulting in intimal hyperplasia and eventual restenosis, or narrowing of the vessel wall [13–17]. This process is further accelerated by pro-inflammatory cytokines released by the activated SMC, including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-8 (IL-8) and interleukin-6 (IL-6) [18–20]. The development of restenosis can lead to peripheral limb ischemia, with treatment often necessitating a second angioplasty procedure to open the re-occluded vessel.

Rather than treating restenosis by attenuating the inflammatory cascade, current surgical methods utilize drug-loaded stents that release cytostatic compounds such as paclitaxel and sirolimus to combat the inflammation-induced cellular proliferation. While they are effective at inhibiting intimal hyperplasia [21–24], these compounds are untargeted and thus inhibit the proliferation of local endothelial cells along with the SMC [22,23]. This results in incomplete healing of the treated area, leaving the stent and collagenous tissue chronically exposed and leading to long-term complications such as late stent thrombosis [25,26]. Additionally, stents deployed in the peripheral vasculature have a high fracture rate due to the higher crushing and torsional forces they experience [27–29]. These issues make drug-eluting stents a suboptimal method to prevent restenosis following angioplasty. Thus there exists a need for an improved treatment system that can be deployed during angioplasty to attenuate the subsequent inflammation and promote healing of the damaged vessel.

We have previously reported on a cell penetrating anti-inflammatory peptide (KAFAKLAARLYRKALARQLGVAA abbreviated KAFAK) that can reduce the expression of pro-inflammatory cytokines, by inhibiting mitogen-activated protein kinase activated protein kinase 2 (MK2) [30]. MK2 increases the production of pro-inflammatory cytokines, such as TNF-α and IL-6, regulating inflammation in several diseases, including atherosclerosis [31–35]. While KAFAK has been effective at reducing pro-inflammatory cytokine levels, poor bioavailability in the presence of serum proteases limits its efficacy [36–38]. Therefore it is desirable to use a delivery system that can protect the peptide from proteolytic degradation while providing a sustained release profile *in vivo*, thereby increasing KAFAK's therapeutic efficacy while providing local delivery to the injured area [38–40].

Poly(N-isopropylacrylamide) (pNIPAM) is a thermosensitive polymer that has been extensively studied in biological applications as it exhibits a physiologically relevant lower critical solution temperature (LCST) around 33 °C [41–47]. At temperatures below the LCST, crosslinked pNIPAM microgels are in a swollen hydrophilic state, allowing for the loading of water-soluble therapeutics via passive diffusion. Above the LCST, the particle then undergoes hydrophobic collapse, entrapping the loaded drug and allowing for a controlled diffusion out of the particle [38,40,47]. In addition, pNIPAM is readily copolymerized with other monomers such as acrylic acid (AAC) to provide easily modified functional groups. Bartlett et al. reported that the addition of the sulfated monomer 2-acrylamido-2-methyl-1-propanesulfonic acid (AMPS) to the NIPAM backbone results in a charged nanoparticle that offers increased loading of cationic therapeutics via electrostatic attraction [38,40]. Additionally, loading the peptide into the nanoparticle provided protection from proteolytic degradation while reducing osteoarthritic inflammation [48]. However these particles were untargeted, allowing them to diffuse away from the site of injury, thus limiting their potential for targeted release. This limitation can be overcome by modifying the delivery system with a targeting ligand that is specific to the damaged area. A particle successfully targeted to the balloon-injured vessel would be able to bind to the damaged area, and remain bound in the presence of blood flow, while locally releasing anti-inflammatory peptides to attenuate the inflammatory cascade.

We have reported on a collagen I binding peptide (RRANAALKAGELYKSILYGC abbreviated SILY) that has been shown to bind to type 1 collagen and prevent platelet adhesion and activation when attached to a dermatan sulfate backbone, while reducing intimal hyperplasia and platelet adhesion *in vivo* [49,50] Additionally, pNIPAM nanoparticles modified with SILY have been shown to bind to collagen and prevent platelet activation while still providing a sustained release of their loaded peptide [51]. However, these studies were conducted in a static environment and did not investigate their efficacy *in vitro*. Here, we show that SILY-modified pNIPAM nanoparticles are effective at inhibiting platelet binding under biologically relevant flow conditions, and are capable of binding to SMC-elaborated collagen *in vitro*. Additionally, KAFAK-loaded nanoparticles effectively inhibit inflammation in endothelial cells (EC) and SMC while inhibiting platelet adhesion and demonstrating minimal cytotoxicity.

## 2. Materials and methods

### 2.1. Nanoparticle synthesis

pNIPAm containing 5 mol% 2-acrylamido-2-methyl-1-propane sulfonic acid (AMPS) and 1 mol% Acrylic Acid (AAc) were synthesized in a precipitation polymerization reaction as described previously [44,51]. Briefly, 794.1 mg of NIPAm (Thermo Fisher Scientific), 28.5 mg of N,N'-methylenebisacrylamide (MBA, Sigma-Aldrich), and 76.5 mg of AMPS (Sigma-Aldrich) were added to 70 °C MilliQ water (18.2 MΩ·cm resistivity, Millipore) that had been refluxed under nitrogen for 30 min. 5 μL of AAc (99.5%, Thermo Fisher Scientific) and 164 μL of sodium dodecyl sulfate (SDS, 10% w/v, Sigma-Aldrich) were added to the solution, and polymerization was initiated by the addition of 33.7 mg of potassium persulfate (Sigma-Aldrich). For fluorescent nanoparticle synthesis, 1 mol% fluorescein o-acrylate was pre-dissolved in 3% DMSO and then added to the shell polymer mixture and allowed to equilibrate for 30 min before the initiation of polymerization. After 5 h, the reaction was cooled to room temperature and then dialyzed against MilliQ water for 7 days using a 15,000 MWCO dialysis membrane (Spectra-Por). Following dialysis, the purified poly(NIPAm-MBA-AMPS-AAc) nanoparticles were lyophilized and stored at room temperature.

### 2.2. Fabrication of peptide-modified nanoparticles

Collagen binding nanoparticles were fabricated by using 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and the heterobifunctional cross linker N-β-Maleimidopropionic acid hydrazide (BMPH) to crosslink SILY to the carboxylic acid groups of present in the nanoparticle, as described previously with slight modification [51]. First, lyophilized poly(NIPAm-MBA-AMPS-AAc) nanoparticles were activated by dissolving them in a coupling buffer consisting of 0.1 M MES (Sigma-Aldrich), 0.5 M NaCl (Sigma-Aldrich) and 0.4 mg/mL EDC (Thermo Fisher Scientific) at pH 6.0. After 15 min, a 1:1 M equivalent of BMPH (BMPH:AAc) was then added to the solution and allowed to shake at room temperature for 30 min. The BMPH conjugated nanoparticles (NP-BMPH) were purified by centrifugation at 18,000*g* and 25 °C for 60 min. The pelleted particles were rinsed with MilliQ water and then lyophilized.

For SILY attachment, lyophilized NP-BMPH were then dissolved at 1 mg/mL in 1X PBS, and 1% of the total concentration of the collagen binding peptide RRANAALKAGELYKSILYGC (SILY, 80% purity,