Nanoenhanced matrix metalloproteinase-responsive delivery vehicles for disease resolution and imaging

Donald A McCarthy¹, Ahmad A Nazem¹, James McNeilan¹, Nicole L Shakerley¹, Ryan R Clark¹, María D Idelchik¹, Mehmet Yigit² and J Andrés Melendez¹

¹SUNY Polytechnic Institute, Colleges of Nanoscale Science and Engineering, Albany, NY 12203, USA; ²University at Albany, Albany, NY 12222, USA

Corresponding author: J Andrés Melendez. Email: jmelendez@sunypoly.edu

Abstract

The wide array of proteases, including matrix metalloproteinases, produced in response to many pathogenic insults, confers a unique proteolytic signature which is often disease specific and provides a potential therapeutic target for drug delivery. Here we propose the use of collagen-based nanoenhanced matrix metalloproteinase-responsive delivery vehicles that display matrix metalloproteinase-specific degradation in diverse *in vitro* models of proteolysis. We demonstrate that collagen particles comprised of protease substrates (primarily collagen) can be made of uniform size and loaded efficiently with assorted cargo including fluorescently labeled mesoporous silica, magnetic nanoparticles, proteins and antioxidants. We also demonstrate that pathologic concentrations of proteases produced *in situ* or *in vitro* display protease-specific cargo release. Additionally, we show that the collagen-based particles display bright fluorescence when loaded with a fluorophore, and have the potential to be used as vehicles for targeted delivery of drugs or imaging agents to regions of high proteolytic activity.

Keywords: Collagen, nanoparticles, matrix metalloproteinase, senescence, antioxidants, infection, burn injury, proteases

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Introduction

Matrix metalloproteinases (MMPs) are extracellular endopeptidases that can have both beneficial and deleterious effects in their environment. The MMP family includes at least 25 zinc-dependent extracellular endopeptidases involved in tissue remodeling.^{1,2} The MMP family members coordinate to modify the extracellular environment, and when unregulated, drive ECM destruction in a wide array of pathologic conditions, such as arthritic disorders, atherosclerotic plaque rupture, chronic obstructive pulmonary disease, metastatic cancer progression, infection, and tissue injury.^{3–8} MMP-1 is a member of the collagenase subfamily of metalloproteinases capable of hydrolyzing the triple helical domain of fibrillar collagen and is required for normal collagen turnover.^{9,10} Once cleaved, other MMP family members including the gelatinases (MMP-2, MMP-9) and stromelysins (MMP-3) further break down collagen. Collagen is a crucial structural protein of connective tissue in mammals¹¹ and is the main component of ECM in humans.¹² There are at least 16 types of collagen, with types I, II and III comprising 80–90% of the collagen in the body.^{13,14} Considering the effects that MMPs have over structural proteins such as collagen, efforts to impair

MMP activity have been naturally targeted for disease intervention. Early work demonstrated that MMP inhibitors (MMPI) were extremely successful in limiting metastatic disease in numerous animal models of cancer.^{15,16} Unfortunately, MMPIs have failed in the clinic as a result of the accompanying musculoskeletal syndrome (MSS) that reduces the overall quality of life for patients.¹⁷ Studies to decrease MMPI concentration to a level that limited MSS failed to display drug efficacy.¹⁷ While efforts have continued to refine MMPI, their use in the clinic has not been forthcoming because MMPs have complex roles and can both be protective or accelerate disease progression. Considering this, an alternative is to harness the proteolytic activity of MMPs for selective therapeutic cargo release that does not target inhibition of MMPs that promote disease resolution. A strategy for utilizing the proteolytic tumor microenvironment through MMP targeting, to achieve higher tumor penetration and increased treatment efficacy, was described by Wong et al.¹⁸ In their design, through activation of endogenous MMPs, a nanoparticle delivery system was degraded into even smaller particles to be able to penetrate deeper into the tumor, thus creating a more effective therapy and reducing systemic side effects. In the same context, we propose a collagen-based

nanoenhanced MMP-responsive delivery vehicle (NMRDV) that allows for selective release of therapeutic or diagnostic agents only at sites of aberrant proteolysis associated with specific disease processes. Taking into account that senescent cells produce nearly 3–5 µg/mL of MMP-1 compared to normal levels between 0.1 and 1 µg/mL,¹⁹ we used this as our MMP-targeting model. Additionally, we will summarize a number of potential therapeutic avenues for the use of NMRDVs and provide experimental evidence for their successful implementation.

Materials and methods

Collagen-based nanoenhanced-MMP responsive delivery vehicle synthesis

In a glass tube, 10 mg of 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine (DMPC) was dissolved in 1 mL of chloroform and vortexed for 2 min. The tube was then purged with nitrogen gas to evaporate the chloroform and create a multilayered lipid on the inside surface of the tube. The multilayered lipid was then rehydrated with a solution of 0.1 mg/mL type I collagen in phosphate buffered saline (PBS) pH 7.4 at 28°C. Where the antioxidant catalase was loaded, the solution was 20 µL of collagen and 980 µL of 2000 U/mL of catalase dissolved in PBS. Collagen was loaded with fluorescent nanoparticles (FNPs) that were either magnetic iron oxide nanoparticles (FeOx-NPs), Green Fluorescent Protein (GFP), or TRITC-labeled mesoporous silica C●spec® Nanoparticles (TRITC-NPs). For Collagen-FNPs, the solution was 20 µL of collagen with 930 µL PBS and 50 µL FNPs. Keeping the solution at 28°C, it was then vortexed for 5 min every 20 min over the course of 1 h. After 1 h, the solution was diluted in 2 mL of PBS and incubated at 37°C for 2 h to polymerize the type I collagen. Following the 2-h incubation, a 60 mM Triton-X solution was added in equal volume (3 mL) to the solution and mixed vigorously to remove the lipid. The solution was then centrifuged for 30 min at 1000 g, pelleting the collagen particles. The pellets were re-suspended in 4 mL PBS followed by centrifugation for 30 min at 1000 g. After the final centrifugation, the pellet was re-suspended in 500 µL PBS.

Cell culture

All cell lines were maintained in 75 cm² flasks at 37°C and incubated at 5% CO₂.

Antioxidant model

Primary human dermal fibroblasts (HDFs) were pre-treated with antioxidant, trolox (100 µM) for 18 h. HDFs were then subjected to a total dose of 120 J (2 J/cm²) of UV-A. Then 24-h post-radiation RNA was extracted to be followed by real-time PCR using MMP-1 specific primers. All values were normalized to the non-treated/radiated control ($n=3$).

Senescent model

IMR-90 primary human fetal lung fibroblasts were cultured in minimal essential medium with 10% FBS. Presenescent

cells were considered from passage < 15 while senescent cells were considered from passage > 25.

Infection model

Mouse lung alveolar macrophage (MH-S) were cultured in RPMI with a final concentration of 0.05 mM of 2-mercaptoethanol and 10% FBS. MH-S cells were plated in cell culture plates at a density of 4×10^5 cells/mL and incubated for 24 h at 37°C. The cells were treated with RPMI, 50 ng/mL, or 150 ng/mL of LPS for an incubation period of 24 h.

Western blot analysis

Samples prepared from various treatments were analyzed for protein concentration using the BCA assay according to the manufacturer's instructions (Pierce/Thermo Scientific, Rockford, IL, USA). Electrophoresis was performed on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred to a nitrocellulose membrane and blocked by incubation for 1 h in 1 × Tween-TBS (TTBS pH 7.6) containing 5% nonfat dry milk. The membranes were incubated with the desired primary antibody in milk with 5% bovine serum albumin (BSA), followed by washing with 1 × TBBS and incubation with the desired dilution of the secondary antibody. Protein detection was performed by the addition of Pierce/Thermo Scientific SuperSignal chemiluminescent substrate for 5 min and exposure to Kodak MS radiographic film (Kodak, Rochester, NY, USA). Data were plotted as the mean relative densitometric intensity via ImageJ and normalized to the loading control. For collagen particles incubated with and without MMP (3 µg/mL) for four days, samples were performed on 10% SDS-Polyacrylamide gels and the above protocol was followed.

Extracellular MMP-1 pulldown

Samples were stored on ice and then spun at 1000 r/min for 10 min at 4°C, with the supernatant being aspirated. Followed by three washes with 1 mL of cold Hank's Balanced Salt Solution (HBSS), 40 µL of mix (150 µL HBSS, 50 µL loading dye, and 5 µL DTT) was added to each tube allowing it be boiled for 10 min. Tubes were spun down at 14,000 r/min for 10 min at 4°C. The supernatant was collected and subsequently electrophoresed.

AMNIS ImageStream

Collagen particles were synthesized and loaded with FNPs or PBS. Data were collected utilizing an ImageStream Imaging Flow Cytometer from Amnis Corp, ~40,000 events and ~10,000 events were collected, respectively, for each sample after gating out debris. Data files were analyzed using IDEAS statistical analysis software (Amnis). Utilizing a 488 laser, bright field and GFP channels were collected.

Scanning electron microscopy

Collagen particles were synthesized and washed $3 \times$ in distilled water. They were then dried on a silicon wafer for enhanced contrast. Once dry, the particles were sputter coated with 5 nm of gold palladium to protect the organic polymer from the electron beam, and subsequently imaged using a Hitachi SEM.

BCA assay

Collagen particles were incubated with 0.1, 0.3, 1 and $3 \mu\text{g/mL}$ of collagenase at 37°C overnight. After incubation, samples were spun down at $10,000 \text{ r/min}$ for 10 min and the supernatant was removed for protein determination following the manufacturers protocol from Pierce/Thermo Scientific, Rockford, IL, USA.

Enhanced darkfield microscopy and hyperspectral imaging

Collagen particles were suspended in PBS, placed onto an ultrasonically cleaned glass slide, and covered with a cover slip. The same process was used for FNP's suspended in PBS. All samples were imaged and analyzed with enhanced darkfield microscopy (EDFM) and hyperspectral imaging (HSI). For EDFM, and Olympus BX-43 microscope with an EDFM condenser was utilized. A CytoViva Hyperspectral Imaging (HSI) Microscopy system (Auburn, AL, USA) was then utilized for hyperspectral analysis through its ENVI 4.8 HSI software. Images were obtained with a DAGE optical camera system for EDFM, and with a Pixelfly camera system for HSI. For scanning and imaging purposes, exposure settings remained constant. First, spectral libraries were created from datacubes (HSI images) obtained from a blank glass slide (negative control), collagen particles, or FNP's alone. Next, the spectral libraries were optimized by filtering against the negative control and pulling spectral lines that were deemed a match. This iterative process was applied to all libraries used in this study. Finally, spectral libraries for collagen particles and for FNP's were mapped against experimental datacubes for content analysis via HSI's feature Spectral Angle Mapping (SAM). All positive mapping results were merged into one representative color and the resulting mapped datacubes were saved as images.

Quantitative RTPCR

Total RNA was extracted from cells using an RNeasy isolation kit (Qiagen) per manufacturer's instructions and stored at -80°C until use. cDNA was synthesized utilizing a Maxima* Universal First Strand cDNA Synthesis kit (Fisher) with random hexamers. Real-time PCR was performed with an Applied Biosystems 7500 real time thermocycler with SYBR green. Specific primers were used for each target: MMP-1, MMP-9, MMP-13 as previously reported in the literatures.^{20,21} PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles of melting at 95°C for 15 s, and annealing and elongation at 60°C for 1 min. Product specificity was determined by melt curve CT values were

normalized to a control housekeeping gene, GAPDH, and the fold change was found using the $\Delta\Delta\text{CT}$ method of analysis.

Statistical analysis

All statistical analyses were performed with the GraphPad 5.04 statistical software package. Statistical significance was assessed using the two-tailed Student's *t*-test or one-way ANOVA with Tukey–Kramer post-test. *P* values of <0.05 were considered significant.

NMRDV cargo release kinetics

To monitor NMRDV cargo release kinetics, we first evaluated the ability of pathologic concentrations of MMP-1 to degrade cargo-free NMRDV or TRITC-NP. To this end, cargo-free and TRITC-NP-loaded NMRDV were synthesized and subsequently incubated overnight with increasing concentrations of recombinant MMP-1 at 37°C . After incubation, intact particles were pelleted and supernatants collected. MMP-1-dependent decay of cargo-free and TRITC-NP NMRDV was monitored using spectrophotometric and fluorescence assays, respectively.

Results

General nanoenhanced MMP-responsive delivery vehicle design

The collagen-based NMRDVs are composed of building blocks that are either biocompatible or generally regarded as safe: MMP substrates are the primary building blocks and can be composed of the many individual collagen types, as well as other matrix components and a diverse array of engineered nanoparticles (Figure 1). The methodologies for the effective production of the NMRDV were based on well-established protocols.²² Cargo loading is

Figure 1 Components of nanoenhanced MMP-responsive delivery vehicle (NMRDV). The image depicts the basic components of NMRDVs. In this study, the matrix components were type I collagen particles, the cargo was either antioxidants (Trolox, Dioxid, pomegranate extract, apple peel extract, catalase) or the MMP1 Ilomostat, and the enhancing FNP's were either GFP-NP, FeOx-NPs, or TRITC-NP. (A color version of this figure is available in the online journal.)

integrated at the collagen polymerization step and release of even small drugs is limited with the majority of cargo released only after protease digestion. Collagen-based NMRDV were produced essentially as described by Swatschek et al.²³ with slight modification to incorporate cargo loading at the rehydration step. Our NMRDVs were type I collagen-based, enhanced with FNP, and loaded with different cargo depending on the experiment. To maintain particle integrity, solutions were normally placed in PBS with the exception of those used for SEM analysis.

During FNP incorporation to NMRDV, particle dye composition was optimized to ensure that there was minimal spectral overlap of fluorescence emission when more than one individual fluorescent particle was combined into a single NMRDV. The carrier particle size is sufficiently large (>50 nm) and the design of the loaded nanoparticle allowed for thorough characterization of release kinetics *in vitro*.

NMRDV characterization

Collagen particles were produced and imaged using SEM (Figure 2(a)). The observed diameter range was between 1 and 20 μm with the majority falling in the 5 μm range. To more quantitatively assess the size distribution and physical characteristics of these particles, they were loaded with FNPs and analyzed using imaging flow cytometry (Figure 2(b)). Over 40,000 events were imaged and characterized, and several physical parameters monitored indicating that NMRDV containing FNPs ranged in size from 1 to 10 μm . Loading of the antioxidant enzyme catalase did not affect circularity (aspect ratio) but did increase NMRDV size which was consistently observed in distinct NMRDV preparations (Figure 2(c)). Collagen-based NMRDV were also loaded with FITC-BSA (Figure 2(d)) and CY5.5 FeOx-NPs (Figure 2(e)). Fluorescence microscopic analyses of FITC-BSA loaded NMRDV support that collagen encapsulates the FITC-BSA (depicted as the corona in merged image Figure 2(e)). Moreover, collagen particles loaded with CY5.5 FeOx-NPs demonstrates cellular enrichment relative to naked CY5.5 FeOx-NPs (Figure 2(e), compare FeOx-NP with Col-FeOx NP).

Hyperspectral properties of FNP and FNP-loaded collagen

CytoViva[®] HSI system allows for analysis of particles and nanomaterials using enhanced darkfield illumination. This enables fast analysis of Rayleigh scatter which provides a unique signature for distinct elemental materials. We used CytoViva[®] to demonstrate the hyperspectral properties of free FNP and FNP-loaded collagen-NMRDVs (Figure 3). Free FNP display darkfield illumination properties that can be clearly distinguished when bulk loaded into the collagen-NMRDV. The SAM feature allowed to determine the presence or absence of FNP and/or collagen particles (Figure 3). Interestingly, FNP-loaded collagen particles appeared to display more uniform polymerization properties. Based on these results, HSI could be potentially used to monitor FNP release and NMRDV deposition in both histological sections and *in vitro* systems.

NMRDV cargo release kinetics in senescent model

To determine if NMRDV can be used to limit the MMP-producing senescent phenotype, we evaluated the cargo release properties of NMRDV comprised entirely of type I collagen in response to collagenase.

Considering that senescent cells produce abnormally high levels of MMP-1 compared to normal cells,¹⁹ cargo release kinetics were evaluated in a senescent model. Increasing concentrations of MMP-1 caused a dose-dependent increase decay of both cargo free and TRITC-NP loaded NMRDV (Figure 4(a) and (b)). We next tested the release of cargo from catalase-loaded NMRDV in response to MMP-1 exposure. Catalase release from protease-treated NMRDV was dramatically enhanced relative to untreated NMRDV (Figure 4(c)).

The above data indicate that the NMRDV is dose-dependently sensitive to pathologic concentrations of purified collagenase. We next evaluated the ability of MMP-1-producing fibroblasts to selectively degrade Catalase-NMRDV and deliver cargo. We chose to load the antioxidant enzyme catalase as we have previously shown that the secretory phenotype of proteolytically active senescent fibroblasts is inhibited by antioxidant treatment.²⁰ Analysis of MMP-1 levels indicates that young or pre-senescent cells show little MMP-1 production when compared to aged or senescent fibroblasts (Figure 5, upper inset). Treatment of young and old cells with catalase-loaded NMRDV indicates that only the aged cells which produce abundant MMP-1 are able to selectively take up catalase (Figure 5, particle-dependent). When catalase was added directly to both cell types, they displayed equivalent levels of uptake (Figure 5, particle-independent). We next inhibited MMP activity using an IC₅₀ of the MMP-1 specific inhibitor Ilomostat and observed a near 50% inhibition of cargo uptake in the aged cells (Figure 5, lower inset). These findings establish the protease-sensing capacity of the NMRDV and that cargo release is dependent on cellular protease activity.

Effects of NMRDV in infection model

The MMPs produced in response to a pathological condition may vary and often display a signature that is disease-specific.²⁴ To test if NMRDVs were capable of responding to proteases produced from infected cells, we evaluated the capacity of bacterial lipopolysaccharide (LPS) activated macrophages to degrade NMRDV in a protease-dependent fashion. We first monitored the MMP-producing capacity of murine alveolar macrophages (MHS) exposed to LPS using RTPCR analysis and immunoblotting. A robust increase in both MMP-9 and MMP-13 from MHS cell lines treated with LPS was observed (Figure 6(a)). We next incubated catalase-NMRDV with media from LPS-treated MHS cells and observed a dose-dependent increase in the release of catalase from the NMRDV when incubated with media from LPS-treated macrophages that was attenuated by the broad spectrum MMP inhibitor, GM6001 (Figure 6(b)). These observations suggest that the infection-induced secretion of MMPs is capable of proteolytically degrading NMRDV and releasing collagen encapsulated cargo.

Figure 2 Physical features and characterization of NMRDV. (a) Scanning Electron Microscopy (SEM) of type I collagen particles dried on a silicon wafer and sputter coated with a layer of gold/palladium alloy. (b) Collagen particles loaded with GFP-labeled BSA were analyzed using imaging flow cytometry and particle size characteristics determined. (c) *Left panel*, Radius of unloaded and catalase loaded particles across different batches. *Right Panel*, Aspect ratios of unloaded (0.817 μm) and catalase-loaded particles (0.7958 μm) averaged ($n = 4$). (d) Immunofluorescent image of FITC-BSA-loaded collagen particles. (e) Cellular distribution of Collagen-FeOx-NPs. IMR-90 primary human fibroblasts Cells were untreated or exposed to FeOx-NPs or Col- FeOx-NPs. Cells were fixed and then probed with FITC-phalloidin DAPI. Immunofluorescence microscopy was performed with the appropriated filter sets to distinguish distinct fluorophores. Actin fibers (green), Nuclei (blue) and Cy5.5 (red). (A color version of this figure is available in the online journal.)

Discussion

Here, we summarize a generalized strategy for the use of NMRDV in targeted drug delivery utilizing disease-specific proteolytic signatures. We report that the high level production of matrix remodeling enzymes such as MMP-1 can be

harnessed for selective cargo release. Similarly, we believe that our strategies for targeting NMRDV towards senescent cells and infections will increase efficacy, decrease systemic toxicity, and increase cargo delivery at proteolytic active disease sites.

Figure 3 Hyperspectral imaging of collagen and FNP-loaded collagen. *Upper panels*, DNV display distinct spectral angle mapping (SAM) characteristics that can be used to interrogate absence or presence of FNP. *Middle panels*, SAM of collagen particles display presence of collagen but not FNP. *Lower panels*, SAM of Collagen-FNP display both collagen and FNP fingerprint. (A color version of this figure is available in the online journal.)

Our findings indicate the feasibility of using a NMRDV to target the proteolytic microenvironment. We have demonstrated that NMRDVs maintain batch to batch uniformity and the ability to load a diverse set of FNPs and proteins as cargo. Additionally, the ability to load catalase into the collagen particles represents an avenue in the use of targeting sites of reactive oxygen species production.

We have demonstrated that to allow for a more localized effect of NMRDVs, the right matrix substrates should be tailored according to the MMP signature of the pathologic condition to be treated. During the NMRDV design, the general approach should be to develop it with differing ratios of the substrate for the MMP to be targeted. For example, if MMP-7 and MMP-13 are the desired protease targets, the NMRDV will be comprised of type I gelatin and type II collagen (4:1, 2:1, 1:1, 1:2, 1:4), respectively. MMP-7 and MMP-13 target a broad array of substrates; however, MMP-13 displays 5-6X more activity toward collagen type II than type I and III.¹ MMP-7 does not readily degrade collagen substrates in the absence of organomercurial activating compounds² and shows preferential activity toward type I gelatin.²⁵ Thus, degradation kinetics can be

determined by monitoring release of FNP with or without added commercially available proteases, and FNP fluorescence would be evaluated by flow cytometric analysis or with the use of microplate based assay systems. We took this approach to evaluate BSA-FITC-loaded collagen-based NMRDV which allowed for rapid assessment of cargo incorporation and size distribution of the particles. FNP fluorescence can be evaluated pre- and post-protease treatment, and fluorescence intensity monitored as a function of time and in response to differing concentrations of protease. MMPs can be added to NMRDV containing FNP, and standard curves are generated based on unincorporated FNP. In the absence of protease digestion the FNP, reported fluorescence is retained in the NMRDV. Incubation of NMRDV with MMPs releases FNP reported fluorescence in an MMP- and collagen-dependent fashion. NMRDV mixtures with more Type II collagen content will display MMP-13-selective degradation with little MMP-7 proteolysis and the reverse for mixtures with high Type I gelatin content. Thus, the appropriate ratio of matrix substrates can be tailored to allow for discrete protease-specific degradation.

Figure 4 NMRDV cargo release properties. (a) Collagen particle degradation was measured by bicinchoninic acid assay (BCA) as relative absorbance of total protein present in supernatant after intact particles were separated out following normalization for collagenase input. (b) Collagen particles were loaded with TRITC-labeled C-specs (Hybrid Silica Technologies) and fluorescence was monitored from supernatants after treatment with the indicated concentrations of collagenase. $n \geq 3 \pm \text{SEM}$, * $P < 0.05$, ** $P < 0.01$ relative to untreated control or as indicated. (c) *Inset*, Catalase-loaded collagen nanoparticles were treated with $3 \mu\text{g/mL}$ collagenase and supernatants collected, and immunoblotting for catalase as indicated. Densitometric quantification of $n \geq 3 \pm \text{SEM}$ representative experiments

The overall objective is to create NMRDV out of a diverse set of protease substrates. Various groups have demonstrated the ability to target diseases through the array of proteases such as MMPs.^{26–28} These modifications allow for more specific targeting by creating requirements for multiple proteases to be present within the tissue for effective cargo release. However, due to the significant degree of MMP substrate overlap, the design will need to go beyond that which occurs naturally. That is, biochemical modifications may be necessary to change the rates at which substrates are degraded by the different types of MMP. This can potentially be accomplished by post-translational bioconjugation methods or by cloning genetic variants that result in

Figure 5 Senescent NMRDV. *Top blot* – Western blot analysis of secreted MMP-1 in pre-senescent and senescent IMR90 fibroblasts. *Middle blots* – Western blot analysis of internalized catalase in pre-senescent and senescent fibroblasts incubated with collagen particles loaded with 500 U/mL recombinant catalase or with 500 U/mL recombinant catalase alone. GAPDH is used as a loading control. *Bottom blot* – Western blot analysis of internalized catalase in senescent fibroblasts incubated with collagen particles loaded with 500 U/mL recombinant catalase in the presence or absence of a broad spectrum MMP inhibitor GM6001 (25 μM). *Lower Graph* – All data normalized to GAPDH controls and expressed as a percentage of maximal loading that was observed in senescent cells as a result of exposure to catalase-loaded particles. Relative densitometric analysis of immunoblots in inset was performed using NIH ImageJ

different three-dimensional folding properties of the collagen fibril. Genetic alterations at the cleavage sites or at the binding sites have the potential to increase proteolytic specificity. Altering the regions on the fibril that MMPs physically associate with may be more useful to this end, since there exists less overlap among the various MMP's binding sites. This will further allow the collagen nanoparticles to be selectively targeted to the area of interest. We refer the reader to recent review on this topic in Vandooren et al.²⁶

Degradation kinetics and cargo release properties of NMRDV can be performed in a cellular or *in vitro* system whose proteolytic signature mirrors that of the disease-specific MMP profile. Nanoparticles have been used extensively for controlled release of pharmaceuticals. However, some iteration is necessary to determine (i) the optimum conditions for adequate FNP loading, (ii) the molar ratios of FNP-collagen conjugate to form stable NMRDV, and (iii) the maximum loading of cargo into the core. The time

Figure 6 Infection NMRDV. (a) qRT-PCR of muMMP-9 and muMMP-13 transcripts from LPS treated and untreated murine alveolar macrophage cell line (MHS). Inset – MMP-13 immunoblot; (b) Catalase–collagen particle degradation in response to supernatants from LPS-treated macrophages. Cargo release is impaired when supernatants are treated with the broad spectrum MMP inhibitor GM6001. Inset, representative immunoblots. Data represent $n \geq 3$. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$ relative to untreated control

scale of proteolytic disintegration of the NMRDV to release the cargo and FNP needs to be determined experimentally. Overall, NMRDVs have the potential to be used for a variety of disease intervention strategies, and several scenarios for their use are described in our experiments and as explained below.

Senescence intervention

Several landmark studies indicate that senescent cells play a causal role in disease, and therapies directed at limiting their activity will restrict severity and onset of many age-associated degenerative diseases.^{29,30} The detrimental activity of senescent cells is attributed to their senescence associated secretory phenotype and the production of interleukins, MMPs and chemokines.^{31,32} We have established that senescence associated MMP expression is regulated by shifts in steady state [H_2O_2] and blocked by limiting metabolic oxidant production or antioxidant treatment.²⁰ The ability of antioxidants to limit age and UV-associated MMP expression has been established by many. Age-associated increases in MMP expression have been attributed to deficits in H_2O_2 detoxification resulting from age-associated decreases in catalase activity.³³ Studies by Haqqi and coworkers have established that both the inflammatory and proteolytic activity of osteoarthritic

chondrocytes can be attenuated by a diverse set of polyphenolic-antioxidant compounds.^{34,35} These observations suggest that antioxidants may prove useful in limiting age-associated disease processes driven by populations of senescent cells. Senescent concentrations of MMP-1 approach those observed in arthritic patients, suggesting this approach may be useful for diseases that are accompanied by high levels of proteolytic activity.^{36,37} It is estimated that more than 70 million baby-boomers will reach retirement in the next 20 years. Thus, compounds that limit degenerative disease have the potential to extend the working lifespan, decrease medical visits, and significantly reduce burden on the rapidly depleting social retirement system. Traditional antioxidant-based therapeutic strategies offer no specificity in terms of delivery and have been shown to have unwanted side effects as exemplified by the failure of several major antioxidant-based prevention strategies to treat disease. It is clear that the cellular-redox state controls cell function and signaling and that the redox-balance can vary in distinct cell types. Thus, it is not surprising that general antioxidant administration fails to limit disease through off-target radical scavenging mechanisms. NMRDV strategies can be used to restrict redox-based activation of the senescence-associated secretory phenotype. The selective antioxidant release NMRDV, would also be suitable for treatment of disease states that involve aberrant MMP-dependent proteolysis, including rheumatoid and osteoarthritic disorders, atherosclerosis, pulmonary fibrosis, periodontal disease, and tumor growth and metastasis.

Infection intervention

MMPs generated in response to any pathogenic insult can vary and often display a disease-specific signature.²⁴ For example, infection of synoviocytes with *Staphylococcus aureus* induces both MMP-1 and MMP-3 while infection with *Brucella abortus* generates a predominant MMP-2 response. The distinct MMP profiles produced by these infectious agents are likely due to the diverse nature of toll-like receptor ligands that are presented by either pathogen. Monitoring the protease fingerprint produced by any individual infection or combination thereof can be used to design personalized NMRDV particles. Thus, in the context of the above bacterial infections, the NMRDV platform can be engineered to match cargo delivery with a particular target infection. This approach would limit emergence of bacterial antibiotic resistance as high concentrations of therapeutic will be delivered only at the focal infectious site.

Limitations

While the studies presented have addressed some hurdles of early stage development, several questions still remain. One potentially problematic possibility is the biodistribution of the NMRDV. For example, NMRDV size may cause them to accumulate in particular regions within the body³⁸ as there exists a clear association with particle size and accumulation in the liver and lung.³⁹ Thus, it remains possible that NMRDV will be rapidly cleared before they have performed their intended function, and thus studies that thoroughly explore these *in vivo* characteristics are lacking,

especially for biologically derived particles. However, nanoparticle biodistribution and pharmacokinetics of drug payloads have been well examined. For example, encapsulating platinum-based anti-cancer drugs can diminish their accumulation in the kidneys, increase their blood circulation time, and increase overall efficacy.^{40,41} Circulation time and organ accumulation of magnetite nanoparticles is similarly affected by encapsulation.⁴² Results like these underline the importance of finding new ways of delivering drugs. Furthermore, encapsulation has the ability to impact the possible routes of delivery. Recent work indicates that orally administered, drug-loaded nanocapsules are more efficiently absorbed by the body.⁴³ Overall, these studies demonstrate the importance of drug delivery systems and our findings suggest that with careful design, delivery systems can be tailored for use in protease targeting.

Conclusions

Here we establish the feasibility of using collagen-based NMRDVs to target proteolytic environments and provide rationale for future studies aimed at tailoring the design and functionality of these delivery vehicles. Ultimately, the use of NMRDV as a platform technology that enables the targeting of a wide array of diseases is desirable. A possible *in vivo* validation of this approach will be pivotal in moving these approaches forward. While we have focused on a select few disease-specific MMP signatures, these approaches have broad applicability. For example, plaque stability is highly dependent on proteases, and NMRDV would serve to target these regions with therapeutics for plaque stabilization. Furthermore, as a diagnostic, NMRDV may be useful for the loading with contrast agents that would allow clinicians to identify regions where potentially dangerous plaques are located.

Many diseases present with unique protease signatures and therefore exploiting a particular signature may open up therapeutic avenues for disease intervention. Coupling a proteolytic targeting strategy with a biological assay capable of detecting a patient's proteolytic signature will reflect translational medicine based on personalized medicine.

Authors' contributions: All authors participated in the design, interpretation of the studies, analysis of the data, and review of the manuscript; DAM, AAN, JM, NLS, and RRC conducted the experiments, MY supplied FeOx nanoparticles. JAM, DAM, AAN, MI, and MY wrote the manuscript.

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protocols, reagents and procedures to conserve resources and to promote health and safety (EHS) in the nanoelectronic- and nanomaterial-associated industries.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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