Therapeutic nanoparticle treatment for acute kidney injury

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ABSTRACT

Cardiac surgery-associated acute kidney injury (CSA-AKI) is a common surgery complication caused by an overproduction of reactive oxygen species (ROS), primarily hydrogen peroxide (H_2O_2), in the body. Despite the prevalence and severity of this condition, there is currently no therapeutic in clinical use for CSA-AKI prevention. APP-103^{TM} is a novel polymer-based nanoparticle designed to quench ROS and prevent damage during surgery, reducing likelihood and severity of AKI. Peroxalate ester bonds along the backbone of PVAX, the active ingredient in APP-103^{TM}, react with ROS at the site of injury to release vanillyl alcohol, an anti-inflammatory. APP-103^{TM} is formed by the emulsification solvent evaporation method, during which PVAX is encapsulated into nanoparticles for intravenous injection. So far, APP-103^{TM} has shown efficacy in mouse and rat models of acute kidney injury, and is under development for clinical application in humans.

Keywords: polymers, nanoparticles, medicine

1 INTRODUCTION

Acute kidney injury can occur as a result of ischemia-reperfusion injury (IRI), when blood flow is interrupted and then reintroduced to the kidneys during an event such as cardiac surgery (CSA-AKI). After a period of limited blood flow and anoxia, organs can get overwhelmed by a flood of reactive oxygen species, such as hydrogen peroxide (H_2O_2), during reperfusion. When this occurs in the kidneys, the resulting AKI can lead to reduced kidney function as well as drastically decrease the likelihood of healthy recovery during a hospital stay.[1] Depending on specific diagnostic criteria, the incidence of AKI from cardiac surgeries is reported between 0.3% and 29.7% and can increase the mortality rate of open heart surgeries by greater than 400%.[2, 3, 4] Even for patients who successfully recover from CSA-AKI, this condition can increase the length of hospital stays and associated costs, as well as lead to longer term kidney health implications.[1] Despite the prevalence and severity of associated negative outcomes, current treatment for AKI focuses on supporting kidney health rather than preventing or mitigating AKI-induced renal damage.

APP-103^{TM} is a nanoparticle therapeutic designed to treat AKI by scavenging hydrogen peroxide and releasing vanillyl alcohol (VA), an anti-inflammatory and anti-apoptotic, at the site of injury. PVAX, the active pharmaceutical ingredient, is composed of a peroxalate backbone with ester bonds connecting vanillyl alcohol. APP-103^{TM}, the formulated nanoparticles themselves, consists of a poly(vinyl-alcohol) (PVA) shell encapsulating a core of PVAX. At the site of high ROS after ischemia reperfusion injury, PVAX reacts with H2O2 to release VA, simultaneously preventing AKI-induced damage by quenching ROS and mitigating existing symptoms and damage.[5]

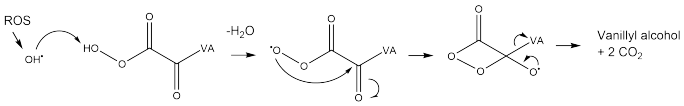

Figure 1: PVAX, active ingredient of APP-103^{TM}, reacts with ROS to release VA

APP-103^{TM} is made by an emulsification-solvent evaporation method, a common and versatile method for the formation of sub-micron particles.[6] In this process, PVA is dissolved into an aqueous solution, while PVAX is dissolved into dichloromethane (DCM), an immiscible solvent. The PVAX solution is then added dropwise into the aqueous solution under high shear, allowing for the PVA to encapsulate and stabilize the PVAX droplets, forming an emulsion. After solvent removal, rinsing, and lyophilization, dry APP-103^{TM} particles remain and can redispersed in water for later use.

2 METHODS

2.1 Nanoparticle Preparation

In the production of APP-103^{TM}, 30g of a 25mg/mL aqueous solution of PVA is prepared by stirring at 80°C for one hour. 17.11g of a PVAX/dichloromethane (DCM) solution is prepared by dissolving 100mg/mL PVAX in DCM by mixing at 40°C for 5 minutes. The

PVAX/DCM solution is then slowly pipetted into the PVA/water solution during high shear vortexing using a Brinkman homogenizer at speed 5. The mixture then undergoes intermittent milling, during which it continues to mix under high shear for five minutes then set to rest for 30 minutes before repeating this process twice. After one final round of mixing, there ultimately will have been three five minute periods of high shear vortexing and two half hour periods of rest. The end result of this process is an emulsion containing droplets of PVAX/DCM encapsulated by PVA.

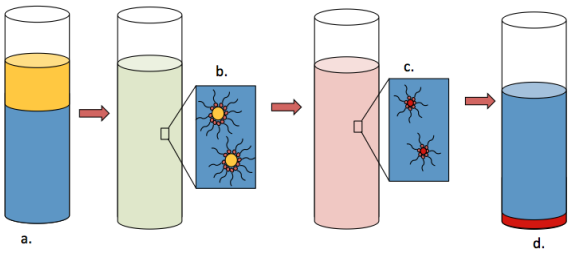

Figure 2: PVAX/DCM solution added to PVA/water solution to undergo emulsification (a), emulsion forms after mixing, with PVA encapsulating PVAX/DCM solution (b), during rotary evaporation, DCM removed from particle centers (c),remaining particles (APP-103^{TM}) settle out of water during centrifugation (d)
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The emulsion then undergoes five minutes of rotary evaporation at 40°C to remove excess DCM before three rounds of centrifuge rinsing at 11,000 RPM and 4°C. The first two rinses are done using distilled water, then during the final round of centrifuge rinsing, 20mL of a solution containing 0.05% Mannitol and 0.5% Tween 20 by weight in distilled water is used. The material is then freeze dried to store at -20°C in powdered form.

Materials used include Mw 89,000-98,000 99+% hydrolized Poly(vinyl-alcohol) from Aldrich Chemistry, D-Mannitol from Tokyo Chemical Industry Co., Tween 20 from Fisher Scientific, and reagent grade (≥99.5% purity) dichloromethane from Fisher Scientific. PVAX was made on commission by Richman Chemical, Inc.

2.2 Characterization

Dynamic light scattering (DLS) and scanning electron microscopy (SEM) are used to verify the size and shape of APP-103^{TM} particles.

2.2.1 Dynamic light scattering

Dynamic light scattering (DLS) is used to measure the radius of APP-103^{TM} particles. A 4 mW helium-neon

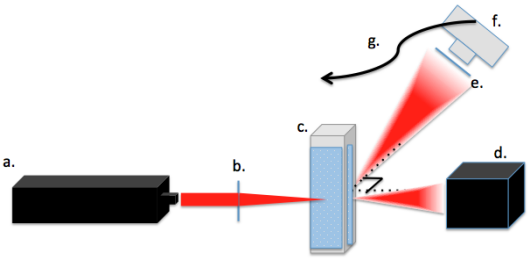

Figure 3: Dynamic Light Scattering schematic. HeNe laser (a), focusing lens (b), sample cuvette (c), beam block (d), laser line interference filter (e), fiber collimator collects scattered light (f), single mode optical fiber (g).

laser with a wavelength of 632.8nm (Research Electro-Optics) is incident on a 1cm^2 cross section cuvette and light scattered perpendicular to the incident beam is collected via a single mode optical fiber into a photon-counting avalanche photodiode (EG&G). The resulting signal is then processed by an ALV5000 (ALV GmbH) autocorrelator before being analyzed with the method described by Frisken[7].

APP-103^{TM} samples are prepared for DLS by dispersing particles at 1mg/mL in PBS by sonicating in water bath for 30 minutes, then diluting to 0.1mg/mL for measurement.

2.2.2 Scanning electron microscope

Scanning electron microscope (SEM) images were taken using a Zeiss SUPRA55-VP. Samples were prepared by re-suspending dried particles in water and dropcasting onto a silicon wafer.

2.3 Hydrogen peroxide assay

A hydrogen peroxide assay is performed using the Pierce Quantitative Peroxide Assay Kit, aqueous-compatible formulation to measure effectiveness of APP-103^{TM} hydrogen peroxide consumption. The assay detects H_2O_2 concentration by peroxide-sensitive changes in UV-vis absorption spectrum of a reagent. To measure the concentration of hydrogen peroxide in a sample, reagent is prepared according to instructions in the Pierce Quantitative Peroxide Assay Kit. One milliliter of reagent is then combined with 100 μL of a sample containing hydrogen peroxide and then an absorption measurement is taken at 560nm using an Agilent Cary 5000 UV-visible absorption spectrometer. Before measuring unknown concentrations, a standard curve is made by measuring absorbance of known concentrations of hydrogen peroxide combined with reagent to obtain an equation for the dependence of absorbance on hydrogen peroxide concentration. All

hydrogen peroxide solutions are prepared by diluting 3% hydrogen peroxide into 0.01M phosphate-buffered saline (PBS).

To prepare samples showing APP-103^{TM} consumption of hydrogen peroxide, APP-103^{TM} is dispersed in PBS at a concentration of 1mg/mL by sonicating in a water bath for 30 minutes. Samples are then prepared by combining various amount of PBS and APP-103^{TM} solution with 1mL of 50-μmolar hydrogen peroxide such that each sample has a total volume of 2mL and initial hydrogen peroxide concentration of 25-μM, with a varying concentration of APP-103^{TM} particles. Control samples are made by combining the same ratios of APP-103^{TM} solution and PBS as in the original samples with 1mL of PBS and hydrogen peroxide, to account for any background due to small amounts of polymer that dissolve during incubation. All samples are then incubated on a shaker for 24 hours at 37°C.

Before measurement, samples are centrifuged at 12,000 RPM for 10 minutes. 100 μL of each sample's supernatant is then combined with reagent for absorption measurements. Analysis is done by subtracting absorbance of control samples from absorbance of samples containing hydrogen peroxide and using the standard curve to calculate the remaining hydrogen peroxide concentration after reaction with the various APP-103^{TM} concentrations.

3 RESULTS

3.1 Particle size and morphology

SEM images and DLS data confirm spherical particles form with an average particle radius of around 400nm. A representative particle size distribution is shown in figure 4. The average particle size for this data (averaged over 20 DLS runs) was found to be 394nm. DLS and SEM data produce results consistent with each other as well as with previous tunneling electron microscopy (TEM).[8]

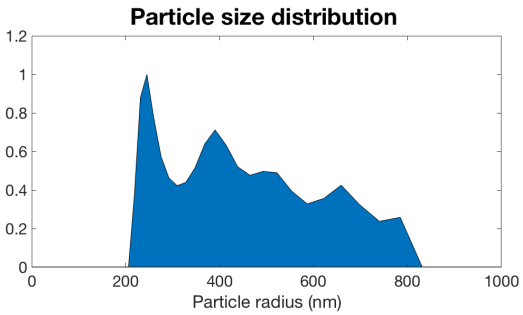

Figure 4: Radius size distribution of APP-103^{TM} dispersed in water, weighted by number of particles.

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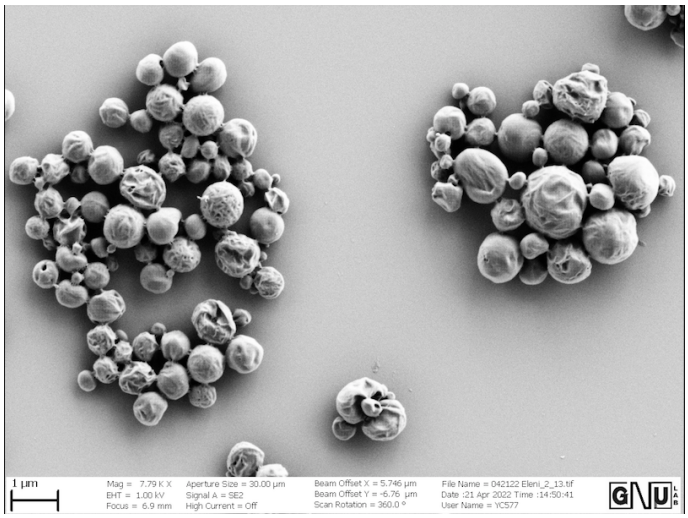

Figure 5: SEM image of APP-103^{TM}

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3.2 Hydrogen peroxide activity

The hydrogen peroxide assay shows that samples with higher concentrations of APP-103^{TM} dispersed in solution with a fixed initial concentration of H_2O_2 yield lower final concentrations of H_2O_2 once background is accounted for, confirming that the particles can successfully scavenge hydrogen peroxide.

Using the calibration curve shown in figure 6, final concentrations of H_2O_2 left in solution for each sample can be approximated, as shown in figure 7. Data is shown from an experiment using three aliquots of each sample testing the efficacy of a single batch of APP-103^{TM}.

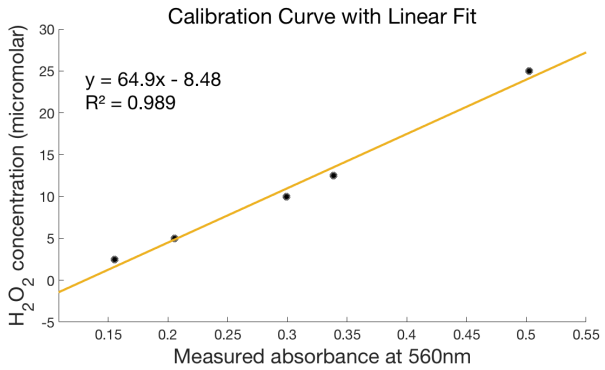

Figure 6: Calibration curve made with known concentrations of hydrogen peroxide. Best fit line is a linear equation y=64.9*x-8.48.

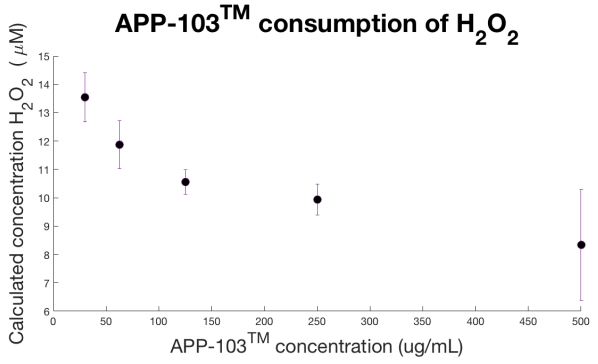

Figure 7: Plot shows calculated values of hydrogen peroxide remaining in solution after reaction with various amount of APP-103^{TM}. Values were calculated using equation from calibration curve,y=64.9*x-8.48, where y is the concentration of H_2O_2 as function of x, the absorbance at 560nm. All samples have initial concentration of 25-μmolar H_2O_2.

3.3 Animal studies

Previous studies have demonstrated efficacy of APP-103^{TM} in various animal models of IRI. In a hind-limb IRI mouse model, ischemia was induced in both hind limbs of mice for 45 minutes before a distal injection of chemiluminescent APP-103^{TM}. Afterwards, one hind limb was reperfused while the other remained ligated as a control. Imaging experiments confirmed the activation of APP-103^{TM} by H2O2 at the site of reperfusion by showing strong chemiluminescent emission at the site of reperfusion when compared to the other limb. Treatment by APP-103^{TM} for hind-limb IRI also showed greater inhibition of inflammation markers and apoptotic myocytes when compared to equivalent doses of VA alone.[9]. This demonstrated the site-specific activation of APP-103^{TM} and provided therapeutic evidence of its ability to counteract IRI. Further, in a renal IRI-induced AKI model, where after 45 minutes of cold ischemic time, APP-103 was administered before the kidneys were reperfused, APP-103^{TM} was again protective against the deleterious effects of AKI, as measured by reduced creatinine levels and inflammation markers 24 hours after IRI and reduced histologically detectable tissue damage 7 days after IRI.[5] Additional models have shown APP-103^{TM} protects against apoptosis and organ damage in both cardiac and liver mouse IRI models.[8]

4 CONCLUSION

Experiments and characterization results confirm the consistent morphology of APP-103^{TM} particles and ability for hydrogen peroxide scavenging, which is associated with VA release. Animal models have proven the efficacy of APP-103^{TM} for mitigation and prevention of IRI in multiple context, in particular showing the therapeutic benefit of APP-103^{TM} in preventing inflammation and tissue damage associated with CSA-AKI. Future work is geared towards scaling up production of APP-103^{TM} and finalizing formulation for future clinical studies

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