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Biodistribution and Renal Clearance of Biocompatible Lung Targeted Poly(ethylene glycol) (PEG) Nanogel Aggregates

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Abstract

A novel stabilized aggregated nanogel particle (SANP) drug delivery system was prepared for injectable passive lung targeting. Gel nanoparticles (GNPs) were synthesized by irreversibly cross-linking 8 Arm PEG thiol with 1,6-Hexane-bis-vinylsulfone (HBVS) in phosphate buffer (PB, pH 7.4) containing 0.1% v/v TweenTM 80. Aggregated nanogel particles (ANPs) were generated by aggregating GNPs to micron-size, which were then stabilized (i.e., SANPs) using a PEG thiol polymer to prevent further growth-aggregation. The size of SANPs, ANPs and GNPs was analyzed using a Coulter counter and transmission electron microscopy (TEM). Stability studies of SANPs were performed at 37 °C in rat plasma, phosphate buffered saline (PBS, pH 7.4) and PB (pH 7.4). SANPs were stable in rat plasma, PBS and PB over 7 days. SANPs were covalently labeled with HiLyteFluorTM750 (DYE-SANPs) to facilitate *ex vivo* imaging. Biodistribution of intravenous DYE-SANPs (30 µm, 4 mg in 500 µL PBS) in male Sprague-Dawley rats was compared to free HiLyteFluorTM750 DYE alone (1 mg in 500 µL PBS) and determined using a Xenogen IVIS®100 Imaging System. Biodistribution studies demonstrated that free DYE was rapidly eliminated from the body by renal filtration, whereas DYE-SANPs accumulated in the lung within 30 minutes and persisted for 48 h. DYE-SANPs were enzymatically degraded to their original principle components (i.e., DYE-PEG-thiol and PEG-VS polymer) and were then eliminated from the body by renal filtration. Histological evaluation using H & E staining and broncho alveolar lavage (BAL) confirmed that these flexible SANPs were not toxic. This suggests that because of their flexible and non-toxic nature, SANPs may be a useful alternative for treating pulmonary diseases such as asthma, pneumonia, tuberculosis and disseminated lung cancer.

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Keywords

Nanogel aggregates; passive pulmonary targeting; drug delivery system; biodegradable microparticle; poly(ethylene glycol)

1. Introduction

Intravenous (IV) administration of microparticles (MPs) larger than the diameter of a capillary offers a unique opportunity for passive drug targeting to the pulmonary circulation [1]. The capillary beds of organs such as the lung, liver, spleen and tumor act as mechanical filters that efficiently entrap MPs following IV administration [1, 2]. Passive lung targeting by IV administration presents an opportunity to treat lung diseases locally from the vascular (perfusion) side thus eliminating the need to traverse the mucus/surfactant and cell/mucosal layers encountered from the airway (ventilation) side. In addition to the cell/mucosal barrier, it is well known that mucus represents a significant diffusional barrier to micron-sized particles [3, 4]. Over the years, a number of reports have been published on the biodistribution of injected particles including poly(lactic-co-glycolic acid) (PLGA) [5, 6], polystyrene [7–9], albumin [10–13] and macroaggregated albumin (MAA) [14, 15], demonstrating their utility for pulmonary drug or vaccine delivery. Since the 1960's, MAA has been utilized in nuclear medicine to image pulmonary blood flow [16]. After IV injection, MAA particles (20–50 μm) accumulate in peripheral lung tissue, and enter the interstitium for eventual clearance by macrophages [17, 18]. Recently, our group reported a highly efficient delivery route via IV injection as an alternative to inhalation for pulmonary drug delivery [1, 2, 19]. Multiple copies of a norvaline (Nva) α -amino acid prodrug of camptothecin (CPT) [20] were attached to the surface of PEGylated 6 μm polystyrene MPs through a degradable ester bond. The MPs were delivered to the capillary bed of the lung following IV administration using a passive size based targeting approach [1]. The study evaluated anticancer efficacy of these MPs in an orthotopic lung cancer model in rats and compared it to a bolus injection of free CPT. Rats receiving free CPT (2 mg/kg) and CPT-Nva-MPs (0.22 mg/kg CPT and 100 mg/kg MPs) were found to have significantly reduced lung cancer areas when compared to untreated animals. In addition, 40% of the animals receiving CPT-Nva-MPs were free of cancer. Although the dose of CPT using targeted MPs was ten times lower than free CPT, it was as effective in reducing the cancer burden than free drug, thus providing encouraging evidence to support the passive lung targeting microemboli approach [1].

Biodistribution and toxicity of MPs depend on their size, rigidity, and dose administered [2]. Additionally, it is imperative that MPs degrade in order to facilitate their elimination after the release of the drug and avoid the accumulation of polymers/delivery vehicles in the body. Thus the development of MPs using non-toxic and non-immunogenic materials is essential. In this study, the design and development of biodegradable stable aggregated nanogel particles (i.e., SANPs) was investigated. We hypothesized that SANPs would be entrapped in the lung due to their size when administered by tail vein injection followed by degradation to the original PEG polymer and elimination by renal filtration because of the significantly reduced PEG monomer size [21, 22].

Aggregation occurs in both biological and chemical systems and this phenomenon is generally considered a negative aspect of working with nanoparticles (NPs) [23, 24]. During the preparation of NPs, many researchers have observed that large unstable aggregates form due to adverse reaction conditions (i.e., high temperature or pH); while concentrating the product [25, 26]; or by various interactions at the molecular level such as hydrophobic and/or ionic interactions [25, 27, 28] of polymers that are commonly used in NPs preparation. In

particular, one of the major challenges associated with NP aggregation is their inability to be resuspended. However, we have used this aggregation phenomenon to create biocompatible lung targeted poly(ethylene glycol) nanogel aggregates.

It is well known that the size and surface properties of particles influence their cellular internalization mechanism, degree of uptake and potential for toxicity [29, 30]. In the current study, we exploit the particle growth by aggregation phenomenon in order to finely control and then stabilize the size of NP aggregates to promote optimal passive targeting to the lung. In addition, programmed renal elimination of the starting PEG-thiol polymer following biodegradation is assessed.

2. Materials and Methods

2.1. Materials

Hexa-glycerine, octa-polyethylene glycol (ether) ethanethiol (8 Arm PEG thiol, MW: 20 kDa) was purchased from NOF America Corporation (White Plains, NY). HiLyte FluorTM750 C2 maleimide dye was purchased from AnaSpec, Inc. (Fremont, CA) and 1,6-Hexane-bis-vinyl sulfone (HBVS) was purchased from (Pierce Biotechnologies Inc., Rockford, IL). Polyoxyethylene (20) sorbitanmonooleate (TweenTM 80) was a generous donation from Croda Inc. (Edison, NJ). All other reagents were purchased from Sigma Aldrich or Fisher Scientific and used without further purification.

Male Sprague-Dawley (200 ± 25 g) rats were obtained from Hilltop Lab Animals, Inc (Scottsdale, PA). Rats were fed a standard diet and provided free access to water. Animals were housed in a room with a 12 h light-dark cycle for at least one week before the study. All rat studies were performed in Association for Assessment and Accreditation of Laboratory Animal Care accredited animal facilities under approved protocols from the Rutgers University Animal Use and Care Committee.

2.2. Preparation of nanogel particles (GNPs)

8 Arm PEG thiol polymer (50 mg, 20 kDa, 1 eq) was dissolved in 3.32 mL PB (pH 7.4). In a separate tube 1,6-Hexane-bis-vinylsulfone (HBVS, 2.5 mg, 1 eq) was dissolved in 1.68 mL PB. The two solutions were mixed and polyoxyethylene (20) sorbitanmonooleate (0.1% v/v, TweenTM 80) added. The reaction mixture was sonicated for 10 min using a probe sonicator (4 watts RMS; MicrosonTM XL 2000, QsonicaLLC., Newtown, CT) with an ice bath and stirred on a magnetic stirrer (Speed 1800 rpm; PC 310, Corning Kent City, MI) at room temperature. After one day, the stirring was terminated and the reaction mixture was frozen at -80 °C for 1 h and then lyophilized for 2 days on a Cascade Freeze Dry System (Labconco Corp., Kansas City, MO; instrument was operating at vacuum 0.010 Bar; collector temperature -81 °C), yielding PEGylated nanogel particles (GNPs). Particle morphology and size were confirmed using TEM and dynamic light scattering (DLS).

2.3. Preparation of aggregated nanogel particles (ANPs)

GNPs were prepared using a procedure similar to the one described above. However the reaction mixture was stirred for 25 days instead of 1 day. After 25 days, stirring was terminated and the reaction mixture lyophilized, yielding ANPs. Particle morphology and size were assessed by TEM and the Coulter counter.

2.4. Stabilization of ANPs

ANPs were stabilized using 8 Arm PEG thiol polymer (20 kDa). PEG-thiol polymer (300 mg) was added into 3 mL solution of ANPs reaction mixture (after 25 days stirring). The solution was agitated slowly using a spatula for 5 min. The agitated sample was lyophilized

as described above (2.2) for 2 days, yielding stabilized ANPs (SANPs). The product was flushed with argon gas and stored at -20°C until further use.

2.5. Preparation of DYE-PEG-thiol

8 Arm PEG thiol (1 eq, 50 mg, 20 kDa) and HiLyte FluorTM 750 C2 maleimide (2 eq, 0.61 mg) were dissolved in DMF (5 mL). The reaction mixture was stirred at room temperature for 30 min, and then added dropwise to pre-cooled diethyl ether (30 mL) to precipitate the product (DYE-PEG-thiol). The product was dried under argon gas and stored at -20°C until further use.

2.6. Stabilization of dye attached SANPs (DYE-SANPs)

ANPs were prepared using the procedure described above. A solution (3.5 mL) of the ANPs reaction mixture was added to 300 mg of DYE-PEG-thiol. The mixture was agitated slowly using a spatula for 5 min and then lyophilized as described above (2.2) for 2 days, yielding stabilized DYE-SANPs as a blue solid. The product was flushed with argon gas and stored at -20°C until further use.

2.7. Preparation of 30 μm SANPs/DYE-SANPs using sonication

To reduce the size of the SANPs/DYE-SANPs (70 μm), SANPs/DYE-SANPs (3 mg) were mixed in (500 μL) sterile saline. The samples were sonicated for 1.5 min at 4 watts (RMS) using a probe sonicator. The particle size was measured using a Coulter counter.

2.8. Dynamic light scattering (DLS)

All dynamic light scattering experiments were performed on a DynaPro-801 (Protein solution Inc., Charlottesville, VA) at 25°C at a wavelength of 830 nm and a scattering angle of 90° . GNPs (1 mg) were weighed and suspended in 500 μL of water by gentle shaking for 30 sec. Resuspended GNPs (20 μL) were loaded into a 1.5-mm cuvette. Data were collected for 20 sec and the data were averaged for three groups of 20 repeats. Data analysis was conducted using DynaPro Instrument Control Software for Molecular Research DYNAMICS (version 5.26.60). Each DLS experiment was repeated in triplicate.

2.9. Particle size analysis

The size of the ANPs and sonicated SANPs were determined using a MultisizerTM 3 Coulter counter (Beckman Coulter, Inc. Miami, FL) with a 560 μm or 100 μm aperture tube. SANPs (100 μL) were added dropwise to 20 mL of ISOTON[®] II dispersed phase until the concentration of particles was acceptable ($<10\%$). One mL of the dispersed phase was counted. Each Coulter counter experiment was repeated in triplicate.

2.10. Transmission electron microscopy (TEM)

Particle morphology and size of GNPs and ANPs were confirmed by TEM. GNPs/ANPs (1 mg) were weighed and suspended in 200 μL of water by gentle shaking for 30 sec. A drop (25 μL) of dispersed ANPs was placed on a 400 mesh carbon coated copper grid. The solution was wicked off the grid and was negatively stained with an aqueous solution of 0.5% uranyl acetate. Grids were analyzed on a Philips CM12 TEM Microscope (FEI, Hillsboro, OR) at 80 kV and images were captured with an AMT digital camera.

2.11. Stability studies of SANPs

The chemical stability of SANPs was assessed in rat plasma, PBS (pH 7.4) and PB (pH 7.4). SANPs (20 mg; size 30 μm) were incubated at 37°C in 2 mL of either rat plasma, PBS or

PB. Aliquots (100 μ L) were collected every day for 8 days. Particle size was measured using a Coulter counter. Stability studies were performed in triplicate (n=3).

Additionally, stability of SANPs (10 mg/mL; size 30 μ m) was assessed by passing through a 24 G needle to check whether the shear stress caused by passing through the syringe and needle will further reduce the SANPs size. Particle size was measured using a Coulter counter. All stability studies were performed in triplicate (n=3).

2.12. Biodistribution studies

Rats ($n = 3$ /group) were anesthetized by 2% isoflurane using an EZ-3500 Multi-Animal Anesthesia System (Euthanex Corp., Palmer, PA). A catheter was then inserted into the tail vein and 500 μ L of either DYE-SANPs (4 mg, 30 μ m) in sterile saline, sterile saline (negative control) or HiLyte FluorTM 750 C2 maleimide dye (1 mg) in sterile saline (positive control) was administered. The catheter was then flushed with 100 μ L of sterile saline. Rats were euthanized by intraperitoneal injection of pentobarbital (250 mg/kg) at 0.5, 6, 18, 24, and 48 h later. The heart, lung, liver, spleen, and kidneys were removed and imaged using a Xenogen IVIS[®] 100 small animal imaging system (Caliper Life Sciences, Hopkinton, MA). The following excitation ($\lambda_{ex} = 710\text{--}760$ nm) and emission ($\lambda_{em} = 810\text{--}875$ nm) filters were used. Identical illumination settings, including exposure time (1 s), binning factor (4), f-stop (2), and fields of view (25×25 cm), were used for all image acquisitions. Fluorescent and photographic images were acquired and merged. The pseudo color image represents the spatial distribution of fluorescence intensity within the organ. Background fluorescence was subtracted prior to analysis. Images were acquired and analyzed using Living Image[®] 2.5 software (Caliper Life Sciences, Hopkinton, MA). The fluorescence signal intensity of each organ was quantified by creating an organ specific, similarly sized circular region of interest (ROI) using of the Living Image[®] 2.5 software. The average efficiency in each experimental group was measured. ROI counts were determined for each time point and the background were subtracted. The ROI signal was normalized by organ weight.

2.13. Confocal microscopy

All images were collected using a Leica TCS SP5 Spectral Confocal Microscope (Leica Microsystems Inc., Buffalo Grove, IL) in the XY mode. The sections were imaged at an excitation wavelength (λ_{ex}) of 633 nm and fluorescence collected at emission wavelengths (λ_{em}) of 660–782 nm.

2.14. Histology

Animals were euthanized by intraperitoneal injection of pentobarbital (250 mg/kg), 48 h after DYE-SANPs exposure. Lungs were perfused with paraformaldehyde (3% in PBS), removed and fixed in paraformaldehyde at 4 $^{\circ}$ C, and then transferred to 50% ethanol. Sections (4 μ m) were prepared, stained with hematoxylin and eosin, and examined by light microscopy. Images were acquired using DP controller software Ver. 3.3.1.292 (Olympus Corporation, Center Valley, PA).

2.15. Bronchoalveolar lavage (BAL) and protein measurement

Animals were deeply anesthetized with Nembutal. A 15-gauge cannula was introduced into the trachea and secured using 3/0 suture. The thoracic cavity was then opened to expose the trachea and lung. The four smaller lobes of the lung were instilled one time with 10 mL of sterile saline at 4 $^{\circ}$ C. The collected lavage fluid was centrifuged at $300 \times g$ for 10 min and the supernatant collected and stored at -80 $^{\circ}$ C until analyses. Total protein content in cell-free BAL was quantified using a BCA protein assay kit (Pierce Biotechnologies Inc.,

Rockford, IL) following the manufacturer's directions with bovine serum albumin as the standard. All samples were assayed in triplicate.

2.16. Detection of renal clearance of SANPs

Animals were euthanized by intraperitoneal injection of pentobarbital (250 mg/kg), 24 h after DYE-SANPs exposure. Urine sample were collected from the bladder (using 24 G syringe needle) and lyophilized as described above (2.2) for 2 days and stored at -80°C until further use. A gel permeation chromatography (GPC) method was used for the analysis and the purification of the urine and nuclear magnetic resonance spectroscopy ($^1\text{H-NMR}$) was used for the characterization of degradation products cleared by the kidneys. Experimental details of GPC and NMR are mentioned in supporting information.

2.17. Statistical analysis

Data were analyzed using GraphPad Prism v4.0c (GraphPad Software, San Diego, CA) and are presented as mean values $\pm$ standard deviation (SD) of three independent measurements. The groups were compared by a two-tail Student's *t*-test, 1-way ANOVA with Tukey's multiple comparison test and 2-way ANOVA with Bonferroni posthoc test.

3. Results and Discussion

To date an alternative drug delivery method that targets the lung passively from the perfusion side (i.e., as contrasted by inhalation or delivery from the ventilation side) has not been extensively investigated. The lung is the only organ in the body that accepts the entire venous blood output from the heart. Thus, large MPs in the venous blood can be trapped in these capillary beds if their size is optimal (i.e., larger than the diameter of a capillary) [2]. This filtering phenomenon can be exploited to selectively deliver MPs to the lung offering a unique opportunity to target the lung. Such delivery methods have been safely employed for the administration of pulmonary perfusion diagnostic agents since the 1960's.

In 1964, human serum albumin "macroaggregates" (MAA) exhibiting suitable size (>90% between 90% of the particles are between 10 and 70 μm , while the typical average size is 20 to 40 μm ; none is greater than 150 μm) and degradation properties were approved for lung targeting by IV injection. Two approved MAA products remain on the market today as pulmonary perfusion diagnostic agents Pulmolite[®] and Draximage[®] [31]. There are a number of degradable polymers that are used for particle preparation for inhalable pulmonary drug or vaccine delivery [5, 6, 10–15]. But to date there are no FDA-approved products that utilize this established and safe pathway for delivering drugs to the lung via intravenous injection.

Large particles (>10 μm) become entrapped in the lungs of humans and rats after intravenous injection. Humans have over 280×10^9 capillary segments with an average diameter of 7–10 μm [32, 33], whereas the diameter of interior pulmonary capillaries in rats are $5.15 \pm 1.3 \mu\text{m}$ [34], suggesting that this passive entrapment philosophy can be tested in rodents. The present studies extend our previous published reports demonstrating that rigid polystyrene MPs ($\sim 6 \mu\text{m}$) become entrapped in the capillaries of the lung after an IV injection [1, 2, 19]. In the current studies, we examine MPs from the other extreme – very flexible particles. Interestingly, IV injected rigid particulates (size <4 μm) pass through the lung and become entrapped by macrophages of the reticuloendothelial system with the majority found in the liver, and the remainder in the spleen and bone marrow [35–37]. Smaller particles (5.5–30 nm) tend to accumulate in a relatively higher concentration in bone-marrow [38]. Particles less than 5.5 nm appear to be completely eliminated from the body via renal clearance [39]. In order to minimize the potential for tissue toxicity or serious

to fatal effects from vascular occlusion (e.g., acute massive embolism, pulmonary edema or the resulting hypoxia), the ultimate goals of this perfusion-based lung targeting are two-fold: (1) utilize particles of optimal size in order to block only the smallest vessels (i.e., capillaries) in the target organ/tissue and not larger upstream vessels, and (2) dose with the fewest particles to avoid vascular occlusion and consequent alterations of microvascular hemodynamics. This would minimize the total number of blocked capillaries and minimize the potential for adverse effects.

Wideman et al. [40] studied the effect of IV injected rigid (silica gel (32–63 μm), polystyrene (15 μm), cellulose (30 μm), SephadexTM (10–80 μm)) MPs on pulmonary hypertension in broiler chickens and cardio-pulmonary hemodynamics. Histological evaluation revealed MPs lodged in inter- and intraparenchymal arterioles, surrounded by aggregates of thrombocytes and mononuclear leukocytes within 30 min MPs injection. They observed that 15 μm polystyrene MPs pass through the terminal arterioles and become trapped within individual capillaries. In this case, only one capillary would be occluded by one MP. Whereas, larger sized MPs such as the 30 μm cellulose MPs, became trapped in the terminal arterial branches. Consequently, each cellulose MP blocked or reduced blood flow through a multitude of “downstream” capillaries since it became occluded in the “upstream” arterial branches rather than the capillaries [40].

3.1. Design and preparation of stabilized aggregated nanogel particles (SANPs)

Michael-type addition reactions have been extensively used in biomedical applications because they occur rapidly without the formation of side products and the reactions proceed under mild reaction conditions [41]. This reaction is quite effective in generating polymer-based bioconjugates with thiol group-terminated polymers, peptides or proteins and it can be accomplished using PEG derivatives modified with vinyl sulfone or maleimide as the preferred end groups [42]. In this transformation thioether linkages were formed at physiological conditions, which are stable and non-degradable [43].

On the basis of these advantages, GNPs and ANPs were formed via a thio-vinylsulfone Michael addition reaction. During the preparation of DYE-SANPs, both vinylsulfone and maleimide polymeric linkages were used. Two-thioether bonds were formed between 8 Arm PEG thiol polymer and HBVS crosslinker (PEG-VS polymer). *In situ* PEG-VS polymer formed the GNPs and ANPs. A third thioether bond was formed between 8 Arm PEG thiol polymer and HiLyteFluorTM750 C2 maleimide dye (DYE-PEG polymer).

SANPs of the desired size were prepared using a two-step formation-size reduction process. 8 Arm PEG thiol (~20 kDa) polymer was irreversibly cross-linked with HBVS in PB (pH 7.4) at room temperature. The two-vinyl sulfone group of HBVS reacted with two thiol groups of 8 Arm PEG thiol polymer via thioether bonds (PEG-VS polymer). Aggregated nanogel particles (ANPs; Figure 1) were formed by aggregating GNPs to micron-size, which were then stabilized using a thiol polymer to prevent further growth. Different experimental conditions were assessed to better understand the effects of stirring time, crosslinker concentration, surfactant and sonication on GNPs size. In each experiment, the reaction mixture was stirred for one day at room temperature using a magnetic stirrer, then lyophilized for two days resulting in GNPs.

The resulting GNP size was determined after varying crosslinker concentration. Smaller hydrogel particles (hydrodynamic radius: 122–125 nm, Supporting information: Figure S1) were generated using either 0.5 eq or 0.8 eq crosslinker concentrations, whereas larger gel particles (hydrodynamic radius: 137 nm) were obtained when 1 eq concentration was used (Supporting information: Figure S1). In order to reduce GNP growth time, larger GNPs (1 eq concentration) were used as a starting material for the ANPs. It is also noted that

sonication for 10 min and the presence of a surfactant (TweenTM 80, 0.1% v/v) were critical for the generation of GNPs. Moreover, either sonication or the presence of surfactant reduced the size of the GNPs (hydrodynamic radius: 10 nm; Supporting information: Figure S2).

The most important factor in GNP generation was stirring time. After stirring the reaction mixture for one day, 10 nm GNPs were formed (Supporting information: Figure S2). If the reaction mixture was stirred for longer periods, the GNPs began to form aggregates with size increasing in proportion to stirring time. To understand the aggregation process, the reaction mixture was stirred for 25 days and aliquots (500 μ L) collected from the reaction mixture once a day and lyophilized. The size of the ANPs was measured by TEM. This analysis revealed that when the reaction mixture was stirred for more than 1 day, aggregates formed as a result of interactions between GNPs. Both small (20 nm) and large (1 μ m) size PEG GNPs/ANPs were detected in the reaction mixture. Figure 2 shows the TEM images of the GNPs/ANPs after stirring the reaction mixture for 1, 6, and 25 days.

Since aggregation continued during stirring, stabilization methods were required to finely tune ANP size. A variety of polymers containing different single or multiple side chain nucleophilic groups (COOH, SH, OH) including poly(ethylene glycol) methyl ether-block poly(ϵ -caprolactone); poly(ethylene glycol) block-poly(lactide methyl ether); poly-L-glutamic acid; α,ω -Bis(2-carboxyl ethyl) poly(ethylene glycol) (20 kDa); and 8 Arm PEG thiol (20 kDa) were investigated for their ability to stabilize the ANPs due to ionic interaction or by reacting with surface reactive groups.

Interestingly, when the thiol groups of the 8 Arm PEG polymer reacted with vinyl sulfone and thiol terminal groups on the particles' surface, the agglomeration process was halted and ANPs were stabilized. The size of the SANPs was 70 μ m as measured using a Coulter counter (Figure 3A and 3C). To employ SANPs for lung targeting and have reduced toxicity, it was necessary to reduce their size in order to minimize blockage of arterioles by larger SANPs after IV administration. This was accomplished by probe sonication for 1.5 min that reduced SANPs size to 30 μ m (Figure 3B and 3C).

3.2 Stability studies of SANPs

SANPs (Size: 30 μ m) were incubated at 37 $^{\circ}$ C in 2 mL of either rat plasma, PBS or PB. Aliquots (100 μ L) were collected at various time points (for 8 days) and particle size was measured using a Coulter counter. Stability studies show that SANPs were found to be stable in rat plasma, PBS (pH 7.4) and PB (pH 7.4) for 8 days at 37 $^{\circ}$ C (Figure 3D). In addition to check the effect of shear force on SANPs particle size, the stability of SANPs (30 μ m) was evaluated by passing them through 24 G syringe needle. SANPs did not disintegrate due to shear force and were considered stable (Figure 3D).

3.3. Lung accumulation and biodistribution studies

For *ex vivo* imaging studies, fluorescently labeled SANPs (DYE-SANPs) were prepared. After stirring for 25 days, aggregates of GNPs were stabilized using HiLyte FluorTM750 C2 maleimide dye attached to PEG-thiol polymer (DYE-PEG-thiol) via a stable thioether bond. The size of DYE-SANPs was reduced to 30 μ m by sonicating the particles.

Rats were intravenously injected with DYE-SANPs (4 mg, 30 μ m) in sterile saline (500 μ L) and free DYE (HiLyte FluorTM750 C2 maleimide, 1 mg in 500 μ L PBS). The biodistribution pattern of DYE-SANPs was determined *ex vivo* by imaging intact organs using a Xenogen IVIS[®]100 Imaging System. These studies demonstrated that free DYE was rapidly eliminated from the body (<30 min), whereas DYE-SANPs accumulate in the lung within 30

min and the majority of the DYE-SANPs remain in the lung for 18 h (Figure 3). Over the course of 48 h, the fluorescent signal in the lung diminished and was corresponding increasingly present in the kidney. The resulting principle components were eliminated by renal filtration and detected in the urine by GPC and NMR.

The average concentration of SANPs per gram of organ weight was demonstrated in Figure 5. The data showed that IV injected SANPs were quickly distributed to the lung. Over the time SANPs degrade in the lung to their original principal components and migrate from the lung to liver then to the kidneys (18, 24 and 48 h). The degradation products are then eliminated from the body via renal filtration.

Lung localization of DYE-SANPs was also confirmed by confocal microscopy. Eighteen hours after injection, DYE-SANPs were mainly located in alveolar wall capillaries (Figure 6). Targeting the pulmonary capillary network allows for a more diffuse and uniform distribution throughout the lung which, in turn, reduces the fraction of overall lung occlusions.

3.4. Characterization and elimination of the degraded SANPs

The degradation products of SANPs were collected from the urine 24 h after IV administration of DYE-SANPs, purified by GPC and characterized using NMR (Supporting information: Figure S3 and S4). At 700 nm GPC spectrum showed the peak for DYE-PEG-thiol at 7.776 min. Whereas, at 220 nm, the peaks for DYE-PEG-thiol and PEG-VS polymer were observed at 7.776 min and 7.937 min, respectively. Both peaks were also observed using an RI detector. NMR spectrum showed that the proton signal for HiLyte Fluor™ 750 dye appeared between 1.0 and 2.5 ppm, and between 7.0 and 7.5 ppm. A proton signal for PEG-VS polymer appeared at 3.27 ppm and 2.6 ppm, whereas proton signals for the PEG polymer that was attached to the dye appeared at 3.38 ppm (Supporting information: Figure S4).

It appears that IV injected SANPs that initially accumulated in the lung subsequently degraded to their original principal components, the DYE-PEG-thiol and PEG-VS polymers. The molecular weight of degraded components is around 20 kDa. Since it is known that the optimal molecular weight of molecules smaller than 40 kDa and hydrodynamic diameter 5.5 nm can easily pass through the kidneys filtering system [39, 44, 45], the renal elimination of the two principal components was expected. GPC and NMR results suggest that DYE-SANPs degrade to their original principle components, the DYE-PEG-thiol and PEG-VS polymer facilitating pre-programmed renal elimination from the body.

There are three thioether bonds that are present in SANPs (i) two thioether bonds between PEG thiol polymer and HBVS crosslinker and (ii) one bond between 8 Arm PEG polymer and HiLyte Fluor™ 750 C2 maleimide dye (DYE-PEG polymer). Since SANPs were stable in PBS, PB and rat plasma, it is speculated that SANPs degradation and elimination from the lung are facilitated through enzymatic degradation. Enzymes such as matrix metalloproteinase-2 (MMP-2) are present in lung [47], and they are constitutively expressed throughout lung development [47–50]. Previously Lévesque et al. [46] reported that thioether bonds specifically degrade due to MMP-2. Therefore, it seems plausible that the *in vivo* degradation of SANPs in the lung occurred by thioether bond cleavage due to MMP-2. The identified degradation products (DYE-PEG-thiol and PEG-VS polymer) are consistent with this mechanism. Interestingly, the GPC and NMR results demonstrated that only one thioether bond (i.e., between PEG thiol polymer and HBVS crosslinker) is cleaved. The other two thioether bonds (i.e., between PEG polymer and HBVS and between the 8 Arm PEG polymer and HiLyte Fluor™ 750 dye) did not degrade. This is likely due to steric hindrance as a result of the presence of the high molecular weight PEG. If the thioether bond

between the PEG polymer and HBVS or between the 8 Arm PEG thiol polymer and HiLyte 750 dye was cleaved, a peak for the 8 Arm PEG thiol polymer would have appeared on the GPC. The peak appears at 8.474 min (Supporting information: Figure S5) but was absent in urine sample (Supporting Information; Figure S3). This finding suggests that only one thioester bond cleavage, which is present between the HBVS and 8 Arm PEG thiol polymers, is the driving force for SANPs degradation and subsequent elimination.

To make SANPs into a viable drug delivery system for passive lung targeting, we are currently pursuing both chemical attachment and physical entrapment methods. For the chemical crosslinking approach, the drug-polymer-linker was prepared by reacting the drug to the 8 Arm PEG thiol (20 kDa) polymer via degradable ester bond. Drug-polymer-linker (20 kDa) was used to stabilize ANPs. Recently, adequate indomethacin surface loading (18%) was achieved by covalently attaching indomethacin drug to SANPs. Physical entrapment offers a different method of drug loading and tailoring drug release and has been widely used in microspheres made of different polymers. These methods also translate to SANPs. A solution or suspension of drug, prodrug or polymeric NP containing a drug would be added during the initial NP formation, the GNP growth phase or the final stabilization of SANPs. In addition, swelling of SANPs to entrap drug may also work, although the pore size of SANPs may be too large to use a water-soluble drug.

Nevertheless, either chemical attachment or physical entrapment methods will be uniquely dependant upon the physical chemical properties of the drug (i.e., water solubility and stability), chemical attachment sites on the polymer or drug and the desired release rates from the SANPs prior to elimination from the lung.

3.5. Lung histology

Lungs were collected 48 h after the injection of control and DYE-SANPs. Lungs were sectioned and sections were stained with hematoxyline & eosin (H & E). No significant changes were observed in lung histology after injection of DYE-SANPs (Figure 7).

Furthermore, additional studies were performed to determine protein levels in BAL as an indicator of alveolar epithelial barrier disruption of lung injuries. There was no significant difference observed in BAL protein levels between the control and SANPs, which was confirmed using two-tailed Student's *t* test (Figure 8).

Based on the histological evaluation and BAL protein assay, it was confirmed that SANPs are not overtly toxic.

4.0 Conclusions

In summary, these results demonstrate that it is feasible to produce stable micron-sized SANPs. SANPs were prepared using a PEG-thiol polymer and an HBVS crosslinker. SANPs were stable in rat plasma, PBS and PB. Biodistribution studies demonstrated that DYE-SANPs selectively accumulated in the lung within 30 min and the majority of the DYE-SANPs remained in the lungs for 18 h. Confocal imaging of DYE-SANPs suggest that localization of these particles occur mainly in alveolar regions of the lung. Histological evaluation using H & E staining and BAL assay confirmed that these flexible and biocompatible SANPs are not overtly toxic. It was observed that enzymatically DYE-SANPs degrade to their original principal components, the DYE-PEG-thiol and PEG-VS polymer and then migrate from the lungs to the kidneys and bladder facilitating elimination from the body by renal filtration. The current study suggests that because of the flexible and non-toxic nature of SANPs, these injectable micron-sized particles may be a useful

alternative to deliver drugs to treat lung diseases including asthma, pneumonia, tuberculosis and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Schematic representation of GNPs, ANPs and SANPs. Gel particles were prepared by irreversibly crosslinking 8 Arm PEG thiol polymer (PEG-thiol polymer) and HBVS crosslinker in PB (pH 7.4) at room temperature.

Figure 2.

TEM images of GNPs and ANPs after stirring the reaction mixture for 1, 6 and 25 days. Gel particles were negatively stained with an aqueous solution of 0.5% uranyl acetate. (scale bars: A and C: 500 nm, B: 100 nm and A inset: 250 nm)

Figure 3.

(A) Size determination of SANPs (70 μm) using Coulter Counter. (B) Size determination of SANPs after 1.5 min sonication (30 μm) using Coulter Counter. (C) Statistical evaluation of the SANPs particle size before and after sonication and after passing through a syringe to simulate injection ($70.000 \pm 0.894 \mu\text{m}$, $30.167 \pm 0.983 \mu\text{m}$, and $31.667 \pm 3.333 \mu\text{m}$, respectively; mean $\pm$ SD, $n=3$). The particle size of pre-sonication of SANPs was statistically different ($p<0.05$) than post-sonication. There was no significant difference between post-sonication particle size and particle size after passing through syringe, which was confirmed using two-tailed Student's t test. (D) Stability studies of SANPs were performed at 37 $^{\circ}\text{C}$ in rat plasma, PBS (pH 7.4) and PB (pH 7.4).

Figure 4.

Biodistribution of DYE-SANPs following IV administration. Rats (n=3) were administered DYE-SANPs (30 µm) through a tail vein catheter. Animals were euthanized by intraperitoneal injection of pentobarbital (250 mg/kg) at 0.5, 6, 18, 24, and 48 h later. The heart, lung, liver, spleen, and kidneys were removed and imaged intact using the IVIS® 100 imaging system.

Figure 5.

The average concentration of SANPs per gram of organ weight. The fluorescence intensity in the lung was statistically different (*, $p < 0.001$), at 0.5 h, 6 h, 18h and 48 h compared to other organs. The significant reduction in fluorescence intensity after 24 h was observed in the lung compared to earlier time points (#, $p < 0.001$), where as no significant difference between the fluorescence intensity was observed (#) at 24 and 48 h. Each column represents the mean $\pm$ SD are shown, ($n = 3$).

Figure 6. Confocal microscopic images of DYE-SANPs in lung tissue. Sections were analyzed at an excitation wavelength 633 nm and emission wavelengths of 660–782 nm. A) Lung control - No particles; B) DYE-SANPs alone; C) Lung section showing DYE-SANPs (30 μm) 18 h post DYE-SANPs injection (scale bar is 50 μm).

Figure 7.

Effects of DYE-SANPs on lung histology. Lung tissue was collected 48 h after injection of control (30 μ m, A and B) or DYE-SANPs (C and D), sections were embedded in paraffin and stained with H & E (Magnification A and C: 100x and B and D: 400x).

Figure 8. Effects of SANPs on BAL protein levels. BAL, collected 24 h after treatment of rats with control or SANPs (4 mg), was assayed for protein content. Each bar is the mean $\pm$ SD (n = 3), mean were not statistically different.