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Poly(vinyl alcohol) Physical Hydrogel Nanoparticles, Not Polymer Solutions, Exert Inhibition of Nitric Oxide Synthesis in Cultured Macrophages

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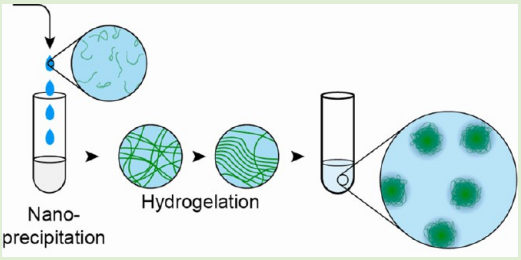
Supporting Information

ABSTRACT: Hydrogel nanoparticles (HNP) are an emerging tool of biomedicine with unique materials characteristics, scope, and utility. These hydrated, soft colloidal carriers can penetrate through voids with dimensions narrower than the size of the particle, provide stabilization for fragile biological cargo and allow diffusion and exchange of solutes with external phase. However, techniques to assemble HNP are few; solitary examples exist of biocompatible polymers being formulated into HNP; and knowledge on the biomedical properties of HNP remains rather cursory. In this work, we investigate assembly of HNP based on a polymer with decades of prominence in the biomedical field, poly(vinyl alcohol), PVA. We develop a novel method for production of PVA HNP through nanoprecipitation-based assembly of polymer nanoparticles and subsequent physical hydrogelation of the polymer. Polymer nanoparticles and HNP were visualized using scanning electron microscopy and fluorescence imaging, and characterized using dynamic light scattering and zeta potential measurements. Interaction of PVA HNP with mammalian cells was investigated using flow cytometry, viability screening, and measurements of nitric oxide production by cultured macrophages. The latter analyses revealed that PVA administered as a polymer solution or in the form of HNP resulted in no measurable increase in production of the inflammation marker. Unexpectedly, PVA HNP exerted a pronounced inhibition of NO synthesis by stimulated macrophages, that is, had an anti-inflammatory activity. This effect was accomplished with a negligible change in the cell viability and was not observed when PVA was administered as a polymer solution. To the best of our knowledge, this is the first observation of inhibition of NO synthesis in macrophages by administered nanoparticles and specifically hydrogel nanoparticles. Taken together, our results present PVA HNP as promising colloidal hydrogel nanocarriers for biomedical applications, specifically drug delivery and assembly of intracellular biosensors.



INTRODUCTION

Development of nanotechnology in preceding decades delivered a materials toolbox for novel therapeutic approaches,[1,2] imaging techniques,[3] and sensing capabilities[4] and, thus, contributed significantly to maturation of biomedicine.[1,5,6] Depending on application, metals, metal oxides, inorganic salts, lipids, polymers, and other materials can be formulated into functional materials with submicrometer dimensions. Of these, hydrogel nanoparticles (HNP) are particularly attractive as carriers for biologically fragile cargo[7,8] and as matrices for creation of intracellular sensors.[9] For drug delivery, a unique characteristic of hydrogels is their capability to deform under pressure and pass through pores with dimensions narrower than the nominal diameter of the particle.[10] This capacity is reminiscent of that of red blood cells and may prove highly important in the context of depth of tissue penetration for drug carrier vehicles. Control over elasticity is also an opportunity available only for hydrogel biomaterials, as is well celebrated for macroscopic biomaterials and established as a powerful tool to control adhesion, proliferation, and differentiation of mammalian cells.[11,12] Early results reveal that particle softness allows controlling the mechanism of cellular entry[13] and, thus, delivers opportunities in defining intracellular trafficking of nanoparticles. For intracellular biosensors,[9] hydrogels are stand-alone candidate materials in that these highly swollen materials allow facile diffusion of solutes and analytes, thus, contributing to the performance of these artificial reporter organelles. Nevertheless, despite the above-mentioned benefits, these opportunities currently receive only a modest research attention, both in fundamental and applied aspects.[7,8] Two factors which are poised to facilitate acceptance of HNP as a tool of biomedicine are (i) development of facile techniques for assembly of HNP and (ii) assembly of HNP using polymers with acceptable level

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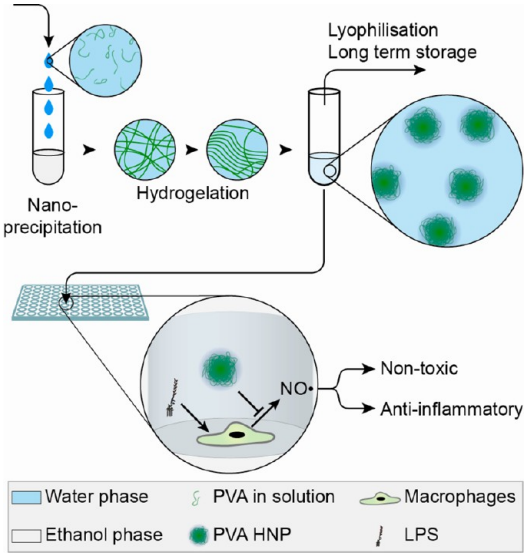
of biocompatibility and regulatory approval for advanced biomedical characterization. With this in mind, we aimed to establish production of HNP based on physical hydrogels of poly(vinyl alcohol), PVA, a polymer with decades of biomedical prominence[14,15] and FDA approval for the use in humans,[16] specifically in a form of embolic bodies.[17] While microparticles[17] and macroscopic gels[15,18] of PVA are well characterized, examples of nanoparticles are solitary.[19−21] With that, successful history of use in biomedicine strongly suggests that HNP based on PVA will prove useful as drug carriers and vessels of biosensors.

Assembly of PVA HNP has previously been accomplished via an emulsion−cryogelation approach[19] and ultrahigh pressure treatment of PVA solutions.[22,23] These two techniques are successful in their own right but leave much room for improvement, specifically with regard to accessibility of instrumentation (high pressure treatment) or uniformity of particles and control over their size (emulsion-based approach). In our recent work, we accomplished assembly of PVA HNP via liposomal templating, that is, producing nanoscopic "droplets" of polymer solution inside liposomes with subsequent hydrogelation to afford particle-shaped hydrogels.[24] Typically, liposomal templating is used to induce hydrogelation inside liposomal voids.[25,26] As a facile alternative, we used liposomes to produce polymer nanoparticles with subsequent removal of the lipids and hydrogelation of lipid-free formulations. Assembled PVA HNP revealed excellent colloidal stability and a remarkable stealth-like behavior, that is, lack of interaction and internalization by mammalian cells, making these colloidal hydrogels promising as long-circulating carriers for diverse biomedical applications. However, liposomal templating and specifically, production of the liposomes via extrusion, exerts significant practical limitations on assembly of PVA HNP through this technique. In particular, encapsulation efficiency of the polymer into liposomes is arrestingly low, overall majority of the polymer chains are not incorporated into the liposomes, and only a minor fraction of the polymer sample is recovered in the form of nanoparticles. Further to this, increasing viscosity of polymer solutions makes extrusion impractical and only low molecular weight samples and/or dilute polymer solutions can be successfully employed. Polymer molecular weight is a characteristic decisive in diverse properties of biomaterials and specifically for PVA, we have shown that physical hydrogels derived thereof exhibit markedly differed elasticity when prepared using polymer samples with varied molecular weights.[27,28] Furthermore, in our recent report we have used molecular weight of PVA as a tool of effective control over spontaneous degradation of physical hydrogels in physiological conditions.[29] Finally, retention and elimination of PVA from the human body is to large extent controlled by the polymer molecular weight.[30] Thus, it is imperative to identify a technique for production of PVA HNP which affords uniform nanoparticles with control over particles size and accommodating polymer samples over a wide range of molecular weights.

In this work, in pursuit of a scalable technique satisfying the above-mentioned criteria, we turned to nanoprecipitation. Success of this tool is verified by its commercial development, specifically for assembly of nanoparticles using organic polymers for drug delivery and vaccination applications.[31] We hypothesized that nanoprecipitation can be used to formulate PVA into nanoparticles thus taking advantage over arms of control made available by this technique; resulting nanoparticles can subsequently be hydrogelated to form HNP using a suit of techniques for chemical and physical cross-linking of PVA (Scheme 1). Herein, we investigate opportunities associated with nanoprecipitation with regard to production of PVA nanoparticles and HNP formulations.

Scheme 1. Schematic Representation of the Scope and Aims of Presented Research[a]



[a]Assembly of PVA nanoparticles was investigated using nanoprecipitation, a facile method which accommodates the use of polymer samples with molecular weight and solution concentrations varied in wide range. Polymer hydrogelation was induced within nanoparticles to obtain well-defined PVA hydrogel nanoparticles (HNP). PVA HNP and parent polymer solutions were administered onto cultured macrophages to investigate cytotoxicity of PVA HNP as well as their pro- and anti-inflammatory effects.

From a different perspective, formulated as nanomaterials, hydrogels become a subject for investigation within the field of nanotoxicology.[5] Microscopic and macroscopic gels are among the most biocompatible materials,[32,33] a notion which has been verified by their utility in tissue engineering and as implantable biomaterials. In contrast, biocompatibility of hydrogel nanoparticles is typically confirmed only through viability assays in cell cultures and as such knowledge on the subject remains rather cursory. It is now understood that biocompatible polymers such as poly(hydroxypropyl methacrylamide) and pluronics may elicit (undesired) intracellular effect once internalized by cells.[34] This includes interference with the performance of intracellular enzyme and proteins (Pgp), activation of nuclear transcription factors (NF-kB) and caspases, and so on.[34] This notion highlights utmost importance of biomedical characterization of nanomaterials extending beyond simple proliferation kits. Specifically, monitoring inflammatory responses mediated by nanomaterials is of particular interest.[5] The second aim of this work was therefore to provide an initial insight into possible intracellular effects elicited by the internalized PVA HNP.

EXPERIMENTAL SECTION

Materials. All chemicals, if not noted otherwise, were purchased from Sigma-Aldrich and used as received. Commercial PVA of varying molecular weights 13−23, 89, and 190 kDa are denoted in this work as low (LMW), medium (MMW), and high (HMW) molecular weight, respectively. Poly(vinyl alcohol-*block*-vinyl pyrrolidone), PVA_{96}-*b*-

PVP_{272}, denoted throughout the manuscript as PVA-*b*-PVP, was synthesized via RAFT polymerization, as described in details previously.[24]

Mammalian cell lines Hep G2 and RAW 264.7 were routinely maintained in Minimum Essential Medium Eagle (MEME, supplemented with Fetal Bovine Serum (FBS, 10%), penicillin streptomycin (P/S, 1%), nonessential amino acids (NEA, 1%), and L-glutamine (2 mM)) and Dulbecco's Modified Eagle's Medium (DMEM, supplemented with FBS (10%) and P/S (1%)). Cells were cultured at 37 °C in a 5% CO_2 atmosphere.

Methods. Dynamic light scattering was performed on a Zetasizer Nano S90, Malvern. Flow cytometry was conducted on a BD Accuri C6 instrument using an excitation wavelength of 488 nm and analyzing at least 1000 events in each sample. Particles and cells were imaged on a Zeiss Axio Observer Z1 fluorescence microscope. Absorbance and fluorescence measurements were conducted using an EnSpire Perkin-Elmer plate reader. High-purity water with a resistivity of 18.2 MΩ/cm was obtained from a Milli Q Direct 8 water purification system.

For SEM imaging, samples were mounted by adhering to the surface of aluminum stubs (Agar Scientific, Stanstead, U.K.) using double-sided carbon tabs (Plano GmbH Wetzlar, Germany). Prior to imaging, the specimens were sputter coated with a conductive layer of Gold/Palladium in a Quorum SC7620 mini sputter coater (Quorum Technologies Ltd., Ashford, U.K.). Samples were then loaded and imaged in a FEI Versa3D SEM (FEI, Hillsboro, U.S.A.) fitted with a field emission gun and operated at an accelerating voltage of 2.0 kV. Images were acquired digitally with 3072 × 2207 pixels and a 10 μs dwell time.

Assembly of PVA Hydrogel Nanoparticles. PVA was dissolved in Milli-Q water first at 90 °C, 300 rpm for at least 3 h, and then equilibrated at 37 °C, 300 rpm overnight, using a thermo-shaker (Eppendorf). Immediately prior to use, the PVA solution was heated at 50 °C for 10 min, and then cooled down to 37 °C. Nanoprecipitation was conducted using polymers with different molecular weight (LMW, MMW, HMW) and solutions with polymer concentrations ranging from 0.25−20 g/L. In a typical experiment, 100 μL of a 5 g/L solution of PVA and containing 0.1 g/L PVA-*b*-PVP was added dropwise to 2 mL ethanol (EtOH) under continuous magnetic stirring (150−200 rpm). Resulting nanoparticles were isolated via centrifugation (10000 rpm, −9 °C, 10 min). Hydrogelation was accomplished via incubation of the nanoparticles in 50 μL 40 v/v% aq. ethanol overnight. To facilitate isolation, suspensions were then supplemented with excess ethanol and PVA HNPs were recovered via centrifugation (10000 rpm, −9 °C, 30 min). The HNPs were dispersed in PBS prior to use or in distilled water when freeze-drying was applied. Assembly of PVA HNP for cell culture experiments was conducted according to the protocol outlined above and using solutions of PVA supplemented with collagen and PVA-FITC to final concentrations of 0.1 g/L and 1 g/L, respectively.

Cellular Uptake, Cytotoxicity, and Nitric Oxide Studies. For cellular uptake studies Hep G2 and RAW 264.7 cells were plated in a 96-well plate (2.5×10^4 cells/well and 1.5×10^4 cells/well for respective cell types) and allowed to adhere overnight in medium. PVA HNP were incubated with the cells for 24 h at 37 °C and 5% CO_2. For analysis the cells were trypsinized with 0.5% trypsin and PVA HNP internalization of the harvested cells was assessed via flow cytometry.

For cytotoxicity measurements of Hep G2 the cells were seeded in a 96-well plate (2.5×10^4 cells/well) for 24 h. PVA HNPs containing 0.1 g/L collagen (30 μg/well) were incubated with the cells for another 24 h, before a PrestoBlue test (Invitrogen) following manufacturer's instructions was performed.

Cytotoxicity and relative nitric oxide (NO) levels in RAW 264.7 cells were assessed simultaneously. The cells were seeded in a 96-well plate (1.5×10^4 cells/well) overnight and subsequently incubated with PVA HNPs (25 μg/well) or PVA solutions (25 μg/well). After 24 h, media was replaced with 90 μL phenol red free complete DMEM media and 10 μL reagent (1 μg/mL lipopolysaccharide (LPS *E. coli* 026:B6)). Cells were incubated with L-N^G-nitroarginine methyl ester (L-NAME, 1 mM), dimethyl sulfoxide (DMSO, 20%), PBS, and only LPS as controls. After another 24 h, a Griess assay[35] was conducted on 50 μL of media and on a 2-fold serial dilution of sodium nitrite (100 − 1.56 μM in media) used to quantify nitrite levels. To start the reaction, 50 μL reagent B (10 g/L sulfanilic acid in 5% phosphoric acid) was added to the samples. After 5 min, 50 μL reagent A (1 g/L *N*-(1-napthyl)ethylenediamine dihydrochloride in water) was added. The absorbance of the samples and the serial diluted sodium nitrite were measured on a plate reader after additionally 5 min at 548 nm. The results of the PVA-HNPs or solutions were normalized to the negative control, incubation with LPS, and reflects the relative NO levels. For the cytotoxicity test remaining media was removed from the cells and a PrestoBlue test was performed.

Cell Fixation and Staining. For visualization of internalized PVA HNP, Hep G2 and RAW 264.7 cells were fixed on glass slides. The glass slides were incubated with 1 mL of 1 g/L PLL in PBS for 1 h prior to cell addition to make the slides cell adhesive. Hep G2 and RAW 264.7 cells were seeded onto the slides (5×10^4 and 1×10^5 cells/well, respectively) in a 12-well plate. The cells were left to adhere for 24 h at 37 °C and 5% CO_2. HNP containing PVA-FITC and 0.1 g/L collagen (dispersed in PBS and diluted with media, ca. 150 μg/well) were added and incubated with the cells for 24 h. The cells were fixed with ethanol. In short media was replaced with 1 mL of a cold 95:5 ethanol:glacial acetic acid mixture for 10 min. The glass slides were washed with PBS three times before addition of 0.5 mL DAPI (100 ng/mL) stain for 30 min. The slides were washed additionally three times with PBS before visualization under the microscope.

Data Analysis. Obtained results were analyzed in Mircosoft Excel 2010 and plotted in OriginPro (Origin Lab, v.8.5). Statistical significance was demonstrated through a two-tailed heteroscedastic T-Test (Excel) and was considered significant if $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

RESULTS AND DISCUSSION

The nanoprecipitation method was originally patented by Fessi et al.[36] This patent includes several guidelines for preparing polymer NPs, one being that the polymer concentration in the solvent should vary between 0.1 and 10% and preferably between 0.2 and 2% by weight. Following this and the methods used by others,[37−39] nanoprecipitation of PVA was examined using aqueous solutions with polymer concentration ranging from 0.25 to 20 g/L and using lower aliphatic alcohols as nonsolvents. Polymer solutions were supplemented with a custom-made block copolymer of PVA with poly(vinyl pyrrolidone), PVA-*b*-PVP, which was necessary to ensure colloidal stability of the formed nanoparticles.[24] In preliminary experiments, we found that ethanol and isopropanol are equally effective in precipitating PVA from its aqueous solutions, and resulting nanoparticles showed a decreased tendency to aggregate in ethanol; for this reason, in subsequent experiments nanoprecipitation was performed using EtOH as a polymer-coagulating solvent. Nanoprecipitation was performed using scintillation vials with 2 mL ethanol as a receiving solution in which case as much as 200 μL aqueous polymer solution could be introduced dropwise without compromising success of technique (data not shown).

Commercial samples of PVA used in this work had molecular weights of 13−23, 89, and 190 kDa, here and below denoted as low (LMW), medium (MMW), and high (HMW) molecular weights, respectively. For each polymer molecular weight, there existed an upper limit in concentration above which nanoprecipitation did not yield nanoparticles but afforded macroscopic polymer precipitation, Figure 1. Thus, for HMW PVA, nanoparticles were obtained using polymer solutions with concentrations not exceeding 5 g/L. With decreasing polymer molecular weight, progressively higher concentrations of polymer solutions could be used for production of nano-

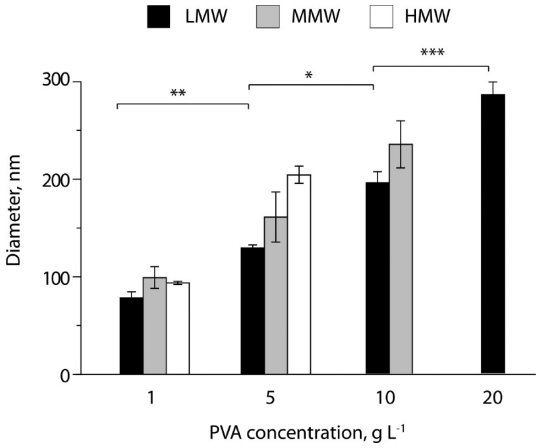


Figure 1. Size of PVA NPs in EtOH after dropwise addition of 100 μL LMW, MMW, and HMW PVA solutions of varied concentrations (1−20 g L^{-1}) containing 0.1 g L^{-1} PVA-*b*-PVP into 2 mL of EtOH, $n = 3$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

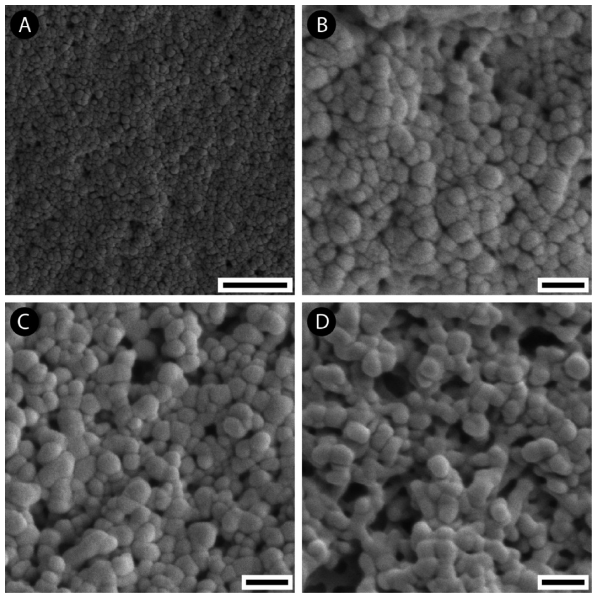


Figure 2. Size of 5 g L^{-1} LMW (A-B), MMW (C), and HMW (D) PVA NPs, scale bars are 1 μm (A) and 200 nm (B−D).

particles and for medium and low molecular weight samples, at least 20 and 10 g/L concentrations, respectively, were suitable for polymer nanoprecipitation. Within the defined range of polymer concentrations, polymer content in the solution used for nanoprecipitation proved to be a powerful tool of control over the size of assembled nanoparticles. This notion is best illustrated on LMW PVA. With decreasing polymer concentration, from 20 to 1 g/L, nanoprecipitation yielded nanoparticles with progressively decreasing sizes, from 300 nm in the case of the highest polymer concentration, to about 200 nm assembled with the use of 10 g/L polymer solution, and below 100 nm for 1 g/L polymer solution. Diluting the polymer to concentrations below 1 g/L did not afford a decrease in particle size. Presented results are based on at least three independent experiments and illustrate good reproducibility of the technique.

Synthesized nanoparticles were collected via centrifugation with typical values of overall polymer recovery of 30−50% by weight. Assembled nanoparticles could be stored for extended periods of time without noticeable signs of aging or aggregation and could be readily redispersed in ethanol to yield suspensions with near identical average size as freshly prepared samples. Samples of NP prepared using 5 g/L polymer solutions were imaged using scanning electron microscopy, Figure 2, which confirmed assembly and isolation of spherical, nonporous particles with sizes corresponding to the values established using dynamic light scattering. Above experiments demonstrate that nanoprecipitation is a facile technique to assemble nanoparticles based on PVA with polymer molecular weight varied in a wide range, from ∼13−23 to 146−186 kDa, and using solutions in a wide range of polymer content. These aspects make nanoprecipitation advantaged over, for example, liposomal templating in that the latter cannot accommodate polymer solutions with high viscosity,[24] which sets the limit on utility of the latter technique to low polymer molecular weights and dilute polymer solutions. Compared to "ultra-high pressure" treatment,[23] nanoprecipitation does not require specialized equipment. In contrast to emulsion-based techniques,[19,40] above developed approach relies on mild, non-denaturing organic solvents and does not employ high shear stresses. All of the above speaks toward practical utility of nanoprecipitation as a method of production of HNP, specifically those based on PVA.

As prepared via precipitation into alcohol solutions, PVA nanoparticles dissolve upon contact with water or aqueous buffered solutions thus revealing insufficient interpolymer binding, that is, lack of physical hydrogelation. Previously, we showed that PVA hydrogelation can be induced via a number of noncryogenic techniques,[28] specifically through incubation in mixtures of water with isopropanol.[24,28] We revealed that pure water and neat IPA afford no hydrogels and PVA samples (microstructured, micrometer thick surface adhered films) dissolved upon contact with phosphate buffered saline. In contrast, water−isopropanol mixtures with 40 vol % content of water elicited hydrogelation of PVA and yielded robust hydrogel samples which remained stable over at least 24 h incubation in physiological media at 37 C.

In this work, we investigated if similar effect can be exerted onto PVA NP and used water − ethanol mixtures with 60 vol % content of alcohol. Preparations of PVA NP were incubated in water−alcohol mixture for 24 h upon which resulting hydrogel nanoparticles were isolated via centrifugation. Prepared samples were directly redispersed in PBS and analyzed using DLS and zeta potential measurements, Figure 3. For each of the three PVA molecular weights (L, M, and H), HNP exhibited polydispersity indexes of <0.2 revealing that nanoprecipitation and noncryogenic hydrogelation afforded well-defined colloidal carriers. Hydrodynamic radii of the HNP were larger than those of the parent PVA NP which is readily explained by characteristic hydrogel swelling (cf. data in Figures 1 and 3). This effect was most pronounced for the LMW sample and only minor for HMW counterpart plausibly revealing higher degree of cross-linking for the latter. For each molecular weight, HNP exhibited a near-zero zeta potential, as would be expected for nanoparticles comprised of this nonionic polymer. Despite this, particles remained colloidally stable and revealed no tendency toward aggregation.

For cell culture experiments, PVA HNP were prepared using a mixture of PVA with collagen, a natural protein with pro-cell adhesive properties. PVA hydrogels have well-documented low

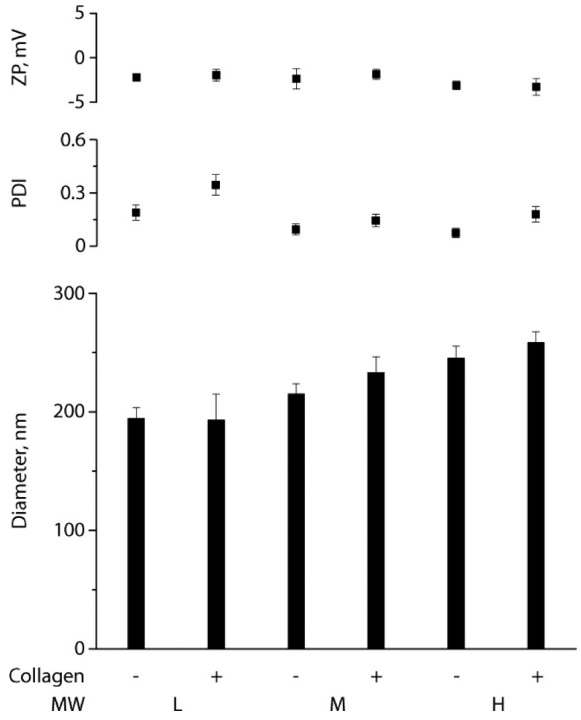


Figure 3. Hydrodynamic diameter, polydispersity index (PDI), and zeta-potential (ZP) of PVA hydrogel nanoparticles prepared via nanoprecipitation and hydrogelation in aqueous ethanol. PVA HNP were prepared using 5 g L^{-1} solutions of polymers optionally supplemented with collagen to 0.1 g/L. Presented results are average of at least three independent experiments and reported as mean ± standard deviation.

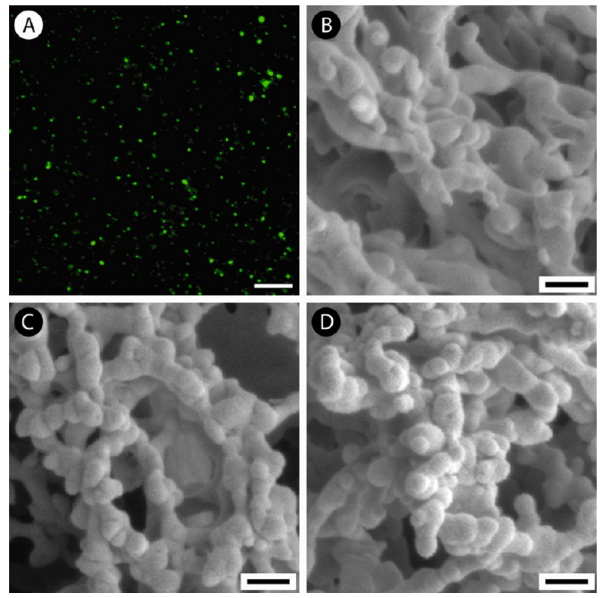


Figure 4. Fluorescence microscopy (A) and SEM (B–D) images of PVA HNP prepared using 5 g/L polymer solutions and samples with low (A, B), medium (C), and high (D) molecular weight. Scale bars are 5 μm (A) and 200 nm (B–D).

cell adhesive properties. In contrast, hydrogels prepared using blends of PVA with collagen (as well as chitosan, poly-L-lysine, etc.) support cell adhesion, and proliferation.[28] We aimed to apply this facile materials design strategy to hydrogel nanoparticles and in assembly of HNP used solutions of PVA containing collagen at a 0.1 g/L concentration. For each PVA molecular weight, collagen-containing HNP exhibited a size similar to pristine, PVA-only counterparts, polydispersity indices at or below 0.3, and near zero zeta potential (Figure 3). In other words, addition of collagen appears to have a negligible effect on the assembly of HNP and the properties of resulting preparation, specifically size and zeta potential.

Visualization of HNP was performed using fluorescence microscopy (FM) and scanning electron microscopy in PBS suspension and dry state, respectively, Figure 4. For FM, samples of HNP were prepared using commercial PVA mixed with custom-made, fluorescently labeled PVA. For the latter, RAFT-derived, amine-terminated PVA was reacted with fluorescein isothiocyanate and purified via precipitation into methanol,[41] as described in detail in the Experimental Section. Resulting preparation of HNP revealed excellent colloidal stability and FM confirmed the expected size of HNP. SEM observations were also consistent with submicrometer-sized nanoparticles. Visual observation suggests that sizes of the particles in the SEM micrographs are lower than the values presented in Figure 3, which is readily explained by HNP dehydration during preparation of the samples for electron microscopy.

With an overall aim of this research focused on the assembly of colloidal drug carriers and hepatic applications, we next examined interaction of PVA HNP with cells belonging to lineages with hepatic relevance, namely, hepatocytes (HepG2) and macrophages (RAW 264.7). For these experiments, colloidal hydrogels were prepared using mixtures of commercial PVA with custom-made, fluorescently labeled polymer. Following 24 h incubation of cells with HNP, analysis of harvested cells was performed using flow cytometry. In this read out, increase in cells fluorescence signifies association and/or internalization of particles by cells. For hepatocytes, 24 h incubation with PVA HNP led to an insignificant increase in cells fluorescence and histogram of distribution of cells fluorescence is similar to that obtained for pristine, nontreated cells, Figure 5A. In contrast, analysis of cells following incubation with hydrogel particles prepared using mixtures of PVA with collagen exhibits a noticeable shift in the histogram suggesting pronounced interaction of the cells with PVA HNP. For macrophages (Figure 5B), incubation with collagen-functionalized PVA HNP also resulted in a significant shift in population of cells to higher fluorescence values. However, in contrast to hepatocytes, incubation with pristine PVA HNP was also accompanied with a pronounced increase in cells fluorescence revealing that for these cells, interaction with HNP does not necessitate the presence of collagen in the hydrogel matrix. Figure 5C,D presents microscopy images for hepatocytes and macrophages following their incubation with fluorescently labeled PVA HNP.

Experiments and analyses presented above were conducted using HNP prepared from samples of PVA with varied molecular weights. Experimental data were used to estimate the fraction of cells which undergo successful interaction with colloidal hydrogels, Figure 5E,F. For both cell lines, interaction of cells with HNP revealed a well-pronounced dose response. This is best illustrated for hepatocytes, in which case 10 μg per well administered dose afforded a negligible fraction of cells testing positive for the associated HNP; in contrast, administration of 50 μg of hydrogel colloids resulted in significantly increased levels of cellular fluorescence which

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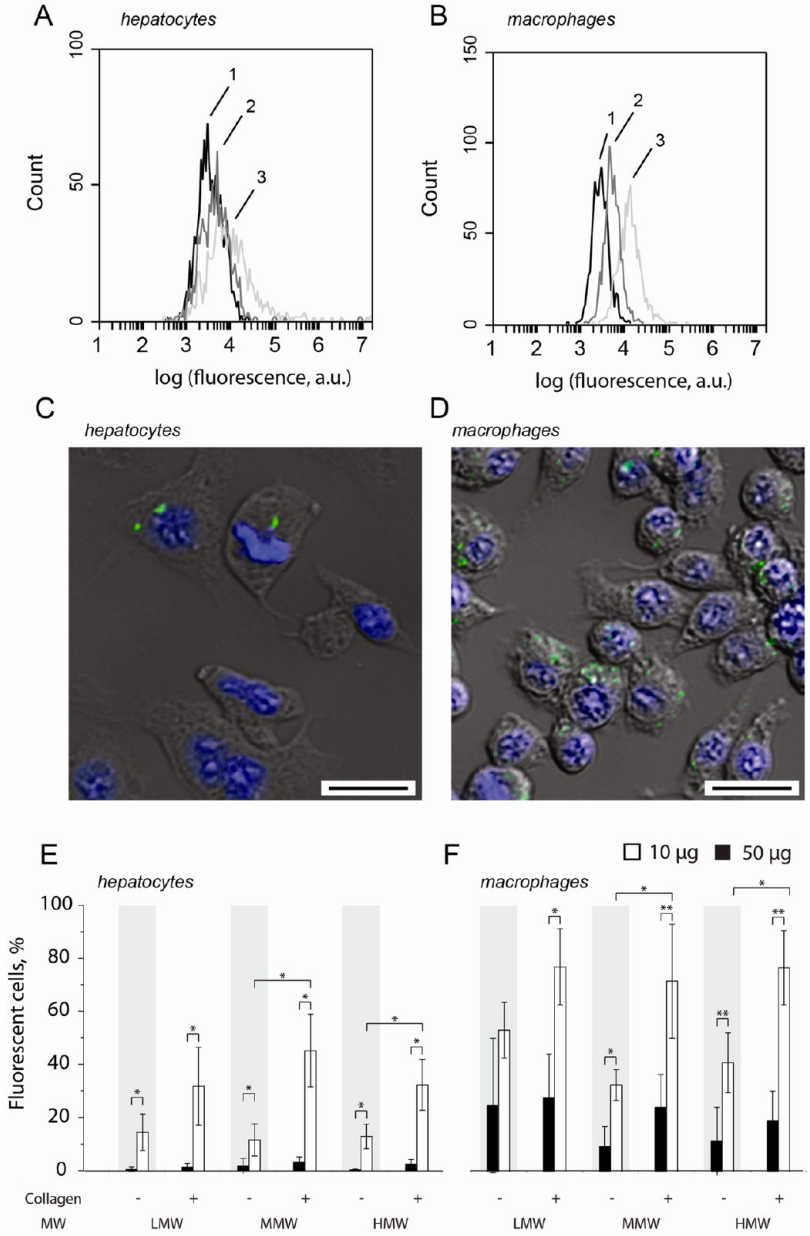

Figure 5. (A, B) Typical flow cytometry histograms obtained for hepatocytes (A) and macrophages (B) upon incubation of cells with PVA HNP for 24 h: pristine cells (1), cells upon incubation with PVA HNPs (2), and cells upon incubation with collagen-containing PVA HNP (3). (C, D) Overlay of differential interference contrast and fluorescence microscopy images for hepatocytes (C) and macrophages (D) upon their incubation with collagen-containing PVA HNP prepared using a 5 g/L solution of PVA with medium molecular weight and supplemented with PVA-FITC (green); cells nuclei were staining using DAPI (blue). (E, F) Percentage of cells testing positive for fluorescence of PVA HNP following a 24 h incubation of hepatocytes (E) and macrophages (F) with PVA HNP. Particles were prepared using solutions of PVA with different molecular weight with polymer concentration of 5 g/L and optionally supplemented with collagen to 0.1 g L^{-1}. Presented results are average of at least three independent experiments and reported as mean ± standard deviation. *P < 0.05, **P < 0.01, ***P < 0.001.

allowed reliable analysis of factors governing interaction of HNP with mammalian cells.

For both cell lines, the presence of collagen within HNP facilitated interaction with mammalian cells, although with a varied degree of statistical significance, as estimated based on three independent experiments. A further important observation is that interaction of HNP with the cells is largely independent of the molecular weight of the polymer used for production of particles. We have previously shown that PVA physical hydrogels prepared using polymer samples with these molecular weights are drastically different in their mechanical properties.[27] Further to this, quantitative data on polymer incorporation into PVA hydrogels revealed that with increased molecular weight, a progressively higher fraction of polymer chains is retained within the hydrogel.[29] Thus, it is highly likely that HNP as used in this study are significantly differed in the polymer content and plausibly softness. The latter parameter is known to significantly affect behavior of mammalian cells at a biomaterials interface[11] and for HNP, matrix elasticity was shown to define the mechanism of internalization by macrophages.[13] Data in Figure 6 illustrate that within the range of molecular weights studied and at least with the current experimental setup, interaction of PVA HNP with hepatocytes

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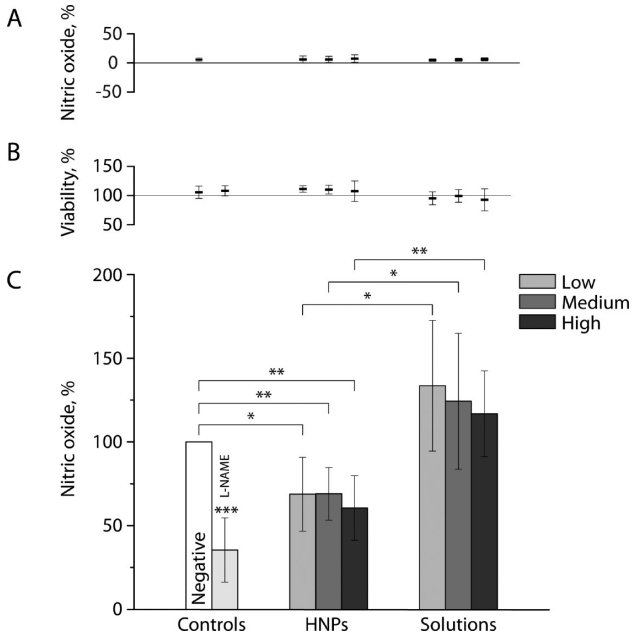

Figure 6. (A) Levels of nitric oxide produced by macrophages upon their culturing in the presence of PVA administered as polymer solution or suspension of HNP. (B, C) Viability and levels of nitric oxide produced by the macrophages upon their culturing in the presence of PVA administered as polymer solution or suspension of HNP and subsequent stimulation with a pro-inflammatory agent, LPS. L-N^G-nitroarginine methyl ester (L-NAME), a potent NO inhibitor was included as a positive control toward anti-inflammatory activity. Presented results are average of at least three independent experiments and reported as mean ± standard deviation. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

and macrophages is not governed by the molecular weight of PVA used in the assembly of hydrogel nanoparticles.

Previously, we showed that interaction of endothelial cells with PVA HNP and their collagen containing counterparts resulted in a minor if any change in the rate of cellular proliferation.[24] To verify this for PVA HNP prepared via nanoprecipitation and using PVA with differed molecular weights, hepatocytes were incubated with HNP over 24 h, and viability assay performed thereafter revealed a negligible change in the cells viability (Figure S1), thus, further supporting the benign, nontoxic nature of PVA HNP as colloidal carriers for biomedical applications.

In as much as macrophages and specifically Kupffer cells (liver resident macrophages) are often the key players in eliminating nanoparticles from circulation, we next aimed to gain a more detailed understanding of the potential effects associated with interaction of PVA HNP with cells of this type. An added objective of this study was to compare effects elicited by polymers administered as solutions or formulated as HNP. Further to a rather cursory viability screening, we monitored pro- and anti-inflammatory activity of PVA, specifically through quantifying levels of nitric oxide produced by the macrophages. NO is a messenger and a therapeutic molecule with diverse functions in the human body.[42−45] Macrophages produce NO in response to diverse pro-inflammatory stimuli, such as viremia, bacterial contamination, and so on. Macrophages cultured on biomaterials also use NO as a way to signal inflammation and, thus, elicit inflammatory response as implicated in tissue acceptance of implants.[46] All of the above makes screening NO levels in cultured macrophages relevant to diverse biomedical applications, from drug delivery to tissue engineering.

PVA polymer and HNP were added at matched concentrations to cultured macrophages. Following 24 h incubation, cells were optionally stimulated by bacterial lipopolysaccharide, a potent pro-inflammatory agent, and levels of NO were quantified using Griess' reagent following a further 24 h incubation. In these experiments, level of NO registered upon stimulation of macrophages in absence of PVA was set to 100%. Unstimulated cells produce NO on a level below detection limit, which corresponds to <10% on the above set scale.

Addition of PVA as polymer solution or formulated as HNP alone and in absence of LPS did not afford increased production of NO and, for all cases, NO levels remained below the level of reliable detection. Possible routes of activation of macrophages toward induced production of inflammatory markers include interaction with receptors on the cell surface, as is documented for nanoparticles,[5] as well as intracellular activation of nuclear transcription factors,[34] for example, NF-κB. The latter phenomenon is well documented for pluronics, amphiphilic, micelle-forming polymers with diverse use in drug delivery.[34] This is highly relevant for presented work in that activation of NF-κB results in an increased synthesis of inducible nitric oxide synthase, an enzyme responsible for production of high concentrations of NO by macrophages. While data in Figure 6 do not rule out activation of nuclear transcription factors, this data set demonstrates that PVA polymer and hydrogel nanoparticles do not elicit production of NO indicative of an inflammatory response.

Unexpectedly and in contrast with an overall majority of nanoparticle formulations reported previously,[5] administration of PVA HNP resulted in a pronounced anti-inflammatory response. Preincubation of macrophages with PVA HNP prior to stimulation with LPS afforded a 25−40% reduction in the levels of NO produced by stimulated macrophages, Figure 6C. Polymer solutions had no such effect; in fact, levels of NO generated by stimulated macrophages following preincubation with polymer solutions were higher than those produced by pristine macrophages, albeit without statistical significance of this observation. For solutions and HNP, administration and incubation with PVA resulted in a negligible change in the viability of cultured macrophages. At the same time, for each polymer molecular weight, inhibition of NO production was statistically significant when compared to LPS-stimulated macrophages and also significant when compared to the same polymer administered in solution phase. Commercially available inhibitor of iNOS (L-NAME) afforded a >50% reduction in the levels of produced NO.

Pro- and anti-inflammatory signals can exert their effect both, from the outside and inside of the cell, that is, act on the surface-localized receptors or intracellular receptors/enzymes.[5] PVA polymer chains are well-known to be low fouling and undergo minimal if any internalization by mammalian cells. In turn, macrophages are professional phagocytic cells which internalize nanoparticles, microparticles, microcapsules, and even cells. Polymers as solutions and HNP were administered at matched doses (in terms of administered polymer mass). Further, taking into account the labile, (bio)degradable nature of PVA physical hydrogels,[29] it is likely that PVA HNP released polymer chains from the hydrogel phase into suspensions over cultured cells. From this, it is possible to conclude that inhibition of production of NO as observed in our experiments

was *not* mediated by PVA polymer chains in solutions. However, this mode of activity cannot be fully ruled out for HNP in which case clustering of chains into nanoparticles may possibly give rise to increased association of particles with cells and receptors on their membranes. From a different perspective, upon cell entry, activity may possibly be exerted by HNP and possibly individual polymer chains released there from as well. The pathway of receptor-mediated activation of macrophages using LPS, subsequent cascade of events leading to activation of NF-κB, increased cellular production of iNOS, and finally the synthesis of NO is very complex; understanding the origin of revealed activity of PVA HNP therefore requires extensive biochemical experiments well exceeding the scope of this work, and this is the subject of ongoing research.

■ CONCLUSIONS

In this work, we developed production of PVA HNP through a combination of nanoprecipitation as a method to produce polymer nanoparticles and subsequent noncryogenic hydrogelation. PVA HNP were assembled using polymer samples with molecular weight varied over a wide range, from as low as 13 to 190 kDa. Assembled hydrogel nanoparticles revealed near zero zeta potential but exhibited excellent colloidal stability. In cultured macrophages, PVA HNP exerted no pro-inflammatory activity but revealed a pronounced anti-inflammatory action manifested as inhibition of production of nitric oxide by stimulated macrophages. We strongly believe that this is the first example of anti-inflammatory activity of hydrogel nanoparticles which renders PVA HNP promising candidates for diverse biomedical applications, specifically drug delivery and assembly of intracellular biosensors.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Data on viability of HepG2 cells in the presence of PVA hydrogel nanoparticles. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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