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Polyamide Nanogels from GRAS Components and Their Toxicity in Mouse Pre-implantation Embryos

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Abstract

Safe delivery systems that can not only encapsulate hydrophobic drug molecules, but also release them in response to specific triggers, are important in several therapeutic and biomedical applications. In this paper, we have designed a nanogel based on molecules that are generally recognized as safe (GRAS). We have shown that the resultant polymeric nanogels exhibit responsive molecular release, and also show high *in vitro* cellular viability on HEK 293T, HeLa, MCF 7 and A549 cell lines. The toxicity of these nanogels was further evaluated with a highly sensitive assay using mouse preimplantation embryo development, where blastocysts were formed after four days of *in vitro* culture and live pups were born when morulae/early blastocysts were transferred to the uteri of surrogate recipients. Our results indicate that these nanogels are non-toxic during mammalian development and do not alter normal growth or early embryo success rate.

Keywords

polymeric nanogel; GRAS; developmental toxicity; redox response

INTRODUCTION

Although the need and scope for the development of drug delivery systems is rather broad, significant attention has been paid to delivery systems for cancer therapy because of the severity and often fatal nature of the disease.¹ With better understanding of the tumor biology, nanocarriers have emerged as a superior class of drug delivery system as they can exploit the leaky vasculature of tumor tissues for selective uptake.^{2–11} Among various classes of nanocarriers, polymeric micelles have attracted particular attention as these can

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Supporting Information Available Additional synthetic details and figures. This material is available at free of charge via the Internet at <http://pubs.acs.org>

noncovalently encapsulate the hydrophobic drug molecules in aqueous conditions.^{12–23} Although polymer micelles show great promise, in many cases these assemblies face a general conundrum with respect to drug loading and encapsulation stability. For high encapsulation stability, it is necessary that the hydrophobic part of the micellar assembly is glassy so as to keep the guest molecules from leaking into the bulk. On the other hand, if the interior of the assembly is too glassy, the loading of drug molecules becomes an issue. The successful utility of polymer micelles, however, in the area of drug delivery has demonstrated that 'sweet spots' can indeed be identified to develop useful nanocarriers. A complementary approach that can offer a viable solution to this issue involves chemically crosslinked polymeric assemblies, where the loading can occur when the assemblies are rather loose and the encapsulation stability is achieved due to the crosslinking-induced incarceration of the drug molecules.^{24–28} Degradable disulfide crosslinked nanogels are fitting illustrations of this possibility, where the crosslinking strategy offers the opportunity to program the assemblies to uncrosslink and release their contents specifically in the presence of a stimulus.^{24–39}

Above all, it is extremely crucial that the drug carrier is safe.⁴⁰ A promising approach to developing such scaffolds is to design the components of the assembly such that they are biodegradable and that the degraded products are non-toxic.^{41–48} In this manuscript, we introduce the design of a stimuli responsive polymeric nanogel system, which can stably encapsulate hydrophobic guest molecules. The polymer is designed such that the degraded products of the polymeric nanogel would be composed of molecules that are generally accepted to be non-toxic. GRAS is the acronym used by the U. S. Food and Drug Administration (FDA) for molecules that are generally recognized as safe; molecules included in the GRAS list can be used as food additives or as medical devices.⁴⁹ Our aim was to synthesize a crosslinked polymeric nanogel using GRAS components which contains disulfide crosslinks. We started with the reasonable assumption that degradation of the polymeric nanogel, under biological conditions, can occur through hydrolysis of esters and amides in addition to the reductive cleavage of the disulfide bonds.⁴⁵ We chose glutamic acid (a naturally occurring amino acid) and putrescine (simplest of the polyamine-based cell cycle regulators) as components of a degradable polyamide backbone. The bifunctional nature of glutamic acid and putrescine was used to synthesize the amide-based polymer backbone. The amino moiety in the glutamic acid was then used as the handle to introduce functional groups that cause self-assembly of this polymer into a nanogel, as well as to incorporate surface functional groups and crosslinkable moieties. Specifically, the polyamide backbone was functionalized with the hydrophilic oligoethyleneglycol (OEG) moiety and the hydrophobic pyridyldisulfide (PDS) moiety. Since this functionalization can render the polymer amphiphilic, the polymer is prone to self-assemble into a nanoscale assembly. This non-covalent, self-assembled structure can be converted to a crosslinked polymer nanogel by employing the recently introduced self-crosslinking strategy where PDS unit is used as the handle.²⁴ Similarly, in addition to providing the hydrophilic component, the OEG moiety also has the potential to endow the nanogel with a surface functionality that is known to endow nanocarriers with reduced non-specific interactions in serum.⁹ Note that the degradation of the side chain functional groups in the nanogel will produce oligoethyleneglycol carboxylic acid and thiopropionic acid, both of which are also

considered to be safe.^{50–51} Structures of the targeted polymer and the nanogel are shown in Schemes 1 and 2.

EXPERIMENTAL SECTION

Materials

All reagents were purchased from commercial sources and used as received, unless otherwise mentioned. ¹H NMR spectra were recorded on a Bruker DPX-400 MHz NMR spectrometer and all the spectra were calibrated against tetramethylsilane (TMS). Dynamic light scattering (DLS) measurements were carried out on a Malvern Nanozetasizer. TEM images were recorded on a JEOL-2000FX machine operating at an accelerating voltage of 100 kV. Cell imaging was performed using a Zeiss 510 META confocal microscope. Fluorescence measurements were performed on a fluorescence plate reader (Molecular Devices, SpectraMax M5).

Synthesis of polymer P1

100 mg (0.226 mmol) of *N*-boc-*L*-glutamic acid di-activated ester was added in a round bottom flask with DMF for stirring. 52.47 μ L (0.376 mmol) of triethylamine was then added to the solution followed by adding 22.7 μ L (0.226 mmol) of 1,4-diaminobutane. The reaction mixture was stirred overnight at ambient temperature, after which the reaction was quenched by cooling down the reaction mixture. The product was purified by precipitation in diethylether followed by dialysis using a 3.5 kDa cut-off membrane in methanol. The product was isolated as an off-white sticky solid. Polymer **P1** was finally synthesized by deprotecting the Boc- moiety through a TFA deprotection (details provided in the Supporting Information).

Synthesis of nanogel precursor polymer P2

75 mg (0.348 mmol) of polymer **P1** and 97 μ L (0.6966 mmol) of triethylamine were dissolved in DMF in a round bottom flask. 113 mg (0.279 mmol) of PEG-NHS **3** and 87 mg (0.279 mmol) of PDS-NHS **2** was then added to the reaction mixture and stirred overnight. The product was then dialyzed in methanol with a 3.5 kDa cut-off membrane (characterization details are shown in Supporting Information).

Synthesis of FITC labeled polymer

A reported procedure was followed with some modification.⁵² 10 mg of polymer **P2** with 85% grafting of PEG and PDS (50:13) was dissolved in 5 mL of methanol in a vial and reacted with a 5 molar excess of Fluorescein isothiocyanate (FITC) by stirring it overnight. The excess non-conjugated FITC was removed by dialysis in methanol with a 3.5 kDa cut-off membrane for 3 days, with constant changing of the bulk solvent till it was free of FITC as observed by fluorescence.

Procedure for hydrophobic dye encapsulation

The polymer **P2** (1 mg/mL) was dissolved in water. 10 μ L (0.01 mg) of 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (1 mg/mL in acetone)

was added to the stirring solution of polymer followed by the desired amount of DTT for crosslinking. The mixture was stirred overnight at room temperature, while being exposed to the atmosphere, allowing the organic solvent to evaporate. Unencapsulated and insoluble excess of DiI was removed by filtration; pyridothione and any unencapsulated/soluble form of DiI were removed from the nanogel solution by dialysis using a membrane with molecular cut-off of 3.5 kDa.

Dynamic light scattering (DLS) study

Dynamic light scattering (DLS) measurements were performed using a Malvern Nanozetisizer with a 637 nm laser source with non-invasive backscattering technology detected at 173°. For the size measurements, the concentration of the polymer and nanogel solution was kept at 1 mg/mL in Milli-Q water. The solution was filtered using a hydrophilic membrane (pore size 0.45 µm) before the experiment was performed. Sizes are reported as the Z-average of the intensity plot and were repeated in triplicate.

Transmission electron microscopy (TEM) study

For the TEM measurements the nanogel solution was prepared in 1 mg/mL concentration. One drop of the sample was dropcasted on carbon-coated Cu grid. About 3 minutes after the deposition, the grid was tapped with filter paper to remove surface water. Finally, it was dried in air for another 6 hours before images were taken.

In vitro cell viability

The *in vitro* cellular viability of the nanogels was evaluated on transformed and cancer cell lines. The cells were cultured in T75 cell culture flasks using Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with 10 % fetal bovine serum (FBS) supplement. The cells were seeded at 10,000 cells per 96-well plate in 200 µL and allowed to grow for 24 hours under incubation at 37 °C and 10 % CO₂. These cells were then treated with nanogels of different concentrations and were incubated for another 24 hours. Cell viability was measured using the Alamar Blue assay with each data point measured in triplicate. Fluorescence measurements were made using the plate reader SpectraMax M5 by setting the excitation wavelength at 560 nm and monitoring emission at 590 nm on a black well plate.

In vitro cell uptake

Cells were incubated overnight at 37 °C and 10% CO₂ with nutrient medium (DMEM/F12 with 10% fetal bovine serum supplement) in glass bottom dishes. The nutrient medium was then taken out and 100 µL of the nanogel solution (10 mg/mL) either encapsulated with 3,3'-diiodo-4,4'-dimethoxydiphenylmethane perchlorate (DiO) or conjugated with FITC were added along with the nutrient medium. The cells were then incubated for 6 hours at 37 °C and the fluorescence was observed under a confocal microscope (63X oil immersion objective).

Degradation of nanogel in serum

1.2 mg of nanogel was incubated in 1.5 mL of fetal bovine serum at 37 °C, 10% CO₂. Before testing the molecular weight by gel permeation chromatography (GPC), 150 µL of

the solution was precipitated in 1.5 mL of cold methanol and the serum proteins were separated by centrifugation. 1.6 mL of the supernatant was then separated and evaporated before analyzing by GPC.

Embryo recovery and culture

B6D2F1 female mice (8 to 10 weeks old) were superovulated with 5 IU pregnant mare's serum gonadotropin (PMSG) and then 5 IU human chorionic gonadotropin (hCG) 46–48 hours later. Females were mated with B6D2F1 males immediately after hCG injection, and euthanized 20–22 hours post-hCG injection. Ampullae were cut open to release 1-cell zygotes and cumulus cells were removed by pipetting in M2 medium containing 0.1% hyaluronidase. Zygotes were randomly divided into groups and cultured in KSOM medium or KSOM supplemented with 1 mg/mL of nanogel solution at 37 °C, 5% CO₂ / 5% O₂ balanced in N₂ for 4 days. Experiments were repeated 3 times, with 2 female mice used for zygote collection. Use of vertebrate animals for embryo production was approved by the University of Massachusetts IACUC.

Embryo transfer

Control (KSOM) and nanogel-treated morulae/early blastocysts were transferred into the uteri of 2.5 dpc (day post coitus) pseudo-pregnant foster dams (CD-1 mice, albino) by non-surgical embryo transfer (NSET). Fifteen embryos from each group were transferred into one recipient (1 female for each group). Recipient females were allowed to deliver pups naturally in order to observe production of live healthy animals after preimplantation development in the presence of nanogel solution.

RESULTS AND DISCUSSIONS

The polymer precursor was synthesized by the reaction between bis-*N*-hydroxysuccinimide ester of *N*-Boc-*L*-glutamic acid and 1,4-diaminobutane (Scheme 2). The resultant copolymer, which was characterized by ¹H NMR and gel permeation chromatography (GPC), was found to have a molecular weight, *M_n* of 8.3 kDa. Removal of the *N*-boc-moiety from the polymer was achieved using trifluoroacetic acid, the conversion of which was quantitative as discerned by ¹H NMR. The amine in polymer **P1** was then treated with equal amounts of the *N*-hydroxysuccinimide esters of oligoethyleneglycol monocarboxylic acid and PDS-protected thiopropionic acid, as shown in Scheme 2. After removing the excess reagents through dialysis, the final conjugation ratio in the target polymer **P2** was determined by ¹H NMR by the characteristic peaks of the PDS moiety at 8.4 ppm and those of the PEG methoxy group at 3.3 ppm (Figure S1). The ratio of these moieties was found to be 7:3 for the hydrophilic PEG and hydrophobic PDS side chain moieties respectively. The difference in the conjugation ratio could possibly be due to the difference in reactivity of the two side chains.

Since the polymer is amphiphilic, it is expected to self-assemble in aqueous solution. This was tested using dynamic light scattering (DLS) and hydrophobic dye encapsulation studies, complemented by transmission electron microscopy (TEM) studies (Figure 1b and 1c). DLS experiments were carried out with a 1 mg/mL solution of polymer **P2** in water and the

aggregates showed a Z-average of 137 nm with a polydispersity of 0.16. Size estimates from TEM were also found to support the DLS measurement. The TEM data also revealed that the assembly has a spherical morphology; the slight departure from a perfectly spherical shape can be attributed to the soft nature of the polymer assembly and the nanogel. Next, we wanted to test if these amphiphilic polymeric aggregates were capable of encapsulating hydrophobic molecules in an aqueous medium. To test this, we used a hydrophobic dye 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) as a fluorescent probe. The dye in itself does not show any absorption in water, as it is hydrophobic and water-insoluble. However, when dissolved in the presence of the polymeric aggregates, its solubility was evident as documented by the absorption spectrum (Figure 1a).

Our next goal was to trap these nanoscale aggregates through chemical crosslinking. We utilized the self-crosslinking strategy in which a sub-stoichiometric amount of dithiothreitol (DTT) was added to the solution containing these aggregates. Briefly, DTT executes a rapid cleavage of the disulfide units from the PDS moieties. Since the pyridothione byproduct is stable and unreactive, the thiol moieties generated in the polymer chain now undergo a thiol-disulfide exchange with the remaining PDS units within the aggregate to cause crosslinked polymeric nanogels.²⁴ The key question is whether the crosslinking reaction is intra-aggregate or inter-aggregate. If it is intra-aggregate, then the size of the crosslinked nanogel should closely correlate with the amphiphilic aggregate from **P2**. Indeed, we found the size of the crosslinked nanogel to be very similar to that of the aggregate (Figure 2b). In generating the nanogels, it is also possible to control the crosslink densities by simply varying the amount of DTT added to the reaction mixture (see supporting information). To test if the extent of crosslinking correlates with the amount of DTT added, we monitored the amount of pyridothione generated in the reaction (Figure 2a). We found a tight correlation between the amount of DTT and pyridothione generation suggesting that DTT-induced cleavage of the PDS units and the subsequent crosslinking reactions are quantitative. We prepared two nanogels; NG1 and NG2 with 8% and 15% crosslink densities respectively (see SI for crosslink density measurement). NG2 was used for all the subsequent studies outlined below.

Once a molecule is encapsulated within the interior, it is also critical that we are able to trigger the release of these molecules in response to a specific stimulus. Since these nanogels contain disulfide crosslinks, they should be responsive to thiol-based reducing environments. Glutathione (GSH) is a reducing agent found in millimolar concentrations in the cytosol, while its concentration in the extracellular environment is micromolar. Thus, we tested the release of the encapsulated dye molecule from the NG2 nanogel scaffold in the presence of 10 mM GSH. We observed that in the presence of GSH, the guest molecule released gradually over time from the nanogel, as discerned from the decrease in the absorption spectrum. The decrease in absorbance of DiI is attributed to the precipitation and settling of the rather hydrophobic guest, upon release from the nanogel due to the GSH-triggered decrosslinking (Figure 3). In a control experiment, where no GSH was added to the solution, the decrease in absorbance was much slower which indicates that the guest molecule release is indeed due to the uncrosslinking of the nanogel scaffold in the presence of GSH.

The key motivation behind designing a polymer system with GRAS components was to design a polymeric nanogel, which exhibits very low toxicity. Accordingly, we first tested the viability of cells grown in the presence of nanogels. Four different cell lines were tested using the Alamar blue assay. NG2 which was incubated along with cell lines for 24 hours at 37 °C showed concentration-independent cellular viability *in vitro* up to a concentration of 0.5 mg/mL (Figure 4). Cytotoxicity studies are meaningful only when the nanoassembly gains access to the cells and still proves not to be cytotoxic. Therefore, we tested whether the nanogels can undergo cellular internalization. Therefore we used nanogels encapsulated with hydrophobic dye, 3,3'-diiodo-4,4'-dimethyldiphenylmethane perchlorate (DiO). DiO nanogels were incubated with HeLa cells for 6 hours and the cellular internalization was evaluated by confocal microscopy. Nanogels clearly enter the cells within the 6-hour culture and are distributed throughout the cytoplasm (Figure S6). It is also possible that the guest molecules can leak from the nanogels, where the hydrophobic dye passively diffuses into the cells. If DiO were to escape the nanogel, it would mainly bind to the cell membrane rather than diffuse into the cytosol.⁵³ To confirm that the DiO signal observed was not due to escape from nanogels, we covalently attached fluorescein to the nanogels and similarly examined them for cellular uptake. Again it is clear that the nanogels were taken up by the cells and uniformly distributed throughout the cytosol (Figure 4).

The non-toxic nature of these nanogels can be attributed to the fact that the degradation byproducts are non-toxic. Although directly testing this hypothesis is difficult, we wanted to determine if the nanogels degrade over time in the presence of serum. We incubated the polymer in serum for several days and evaluated it by gel permeation chromatography. We found gradual increase in the elution time over a period of 6 days, suggesting that the molecular weight of the polymer decreases over time (Figure S7). This is a preliminary indicator that the nanogels will indeed degrade in serum conditions. However, detailed evaluation of the degradation products requires further investigation.

After toxicity assessment in somatic cells, the nanogels were further evaluated using a more sensitive model – co-culture with preimplantation embryos. Mammalian preimplantation development is a time of dynamic change in which the fertilized egg undergoes cleavage divisions and subsequent development into a blastocyst stage embryo. Many essential cellular events occur during this time including DNA replication, epigenetic reprogramming, zygotic genome activation, blastomere allocation and cell lineage specification.⁵⁴ Preimplantation embryos are highly sensitive to environmental changes and perturbations which can impair the dynamic morphogenetic events resulting in failure of blastocyst formation and/or long-term embryo survival.^{54–56} It has been suggested that preimplantation embryos are generally more sensitive to toxicants than somatic cells.^{57–58} We therefore assessed development of embryos in the presence of nanogels. Zygotes were cultured in KSOM with or without FITC-labeled nanogels (KSOM+NG, Figure 5). Embryos were carefully monitored for developmental progress. No significant differences were observed during preimplantation development in the presence of nanogels. 65 of 73 control zygotes formed blastocysts and 64 of 72 zygotes in KSOM+NG formed blastocysts. In addition, fluorescence signal was detected in KSOM+NG blastocysts, but not in the KSOM controls (Figure 6) indicating that the FITC labeled nanogel was taken up by blastomeres during preimplantation development, but did not alter developmental potential of embryos. These

results show that the nanogels are not toxic to mammalian preimplantation embryos and pluripotent cells *in vivo*.

To further investigate the toxicity and rule out the possibility that nanogel exposure may cause long-term defects not detected during the preimplantation period, embryos were transferred to pseudo-pregnant recipients after nanogel exposure in culture. Live healthy pups were born after preimplantation development in the presence of these glutamic acid nanogels (Figure 6C'' and 6D'') indicating that multiple-day exposure is compatible with long-term survival. Taken together, our results indicate that the nanogels are non-toxic to mammalian preimplantation development and that fetal development after culture in and uptake of nanogels is similarly unaffected, permitting normal growth and survival to birth.

CONCLUSIONS

In summary, we have developed a new polyamide nanogel, the building blocks of which are based on components that are generally regarded as safe. The backbone of the polyamide is based on glutamic acid and putrescine, while the side chain substituents are based on oligoethyleneglycol and thiopropionic acid. Since the precursor to these side-chain substituents make the polyamide amphiphilic, the polymer self-assembles in aqueous phase. Disulfide crosslinked polymeric nanogels have been obtained using this self-assembly, while concurrently taking advantage of the amphiphilic nature of the assembly to sequester hydrophobic molecules within its interior. Since the crosslinks are based on disulfide functionalities, these nanogels exhibit molecular release in response to the intracellular stimulus, glutathione. Finally, to test the versatility of the biocompatible nanogel design, nanogels were tested for toxicity using an *in vitro* tissue culture cytotoxicity assay as well as a more rigorous and highly sensitive mammalian preimplantation development assay. In neither assay do the nanogels exhibit discernible toxicity, indicating that these GRAS-based stimuli responsive nanogels have great potential for *in vivo* applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Self-assembly of polymeric aggregates as seen by a) hydrophobic guest encapsulation where DiI encapsulated in the polymer shows an absorbance in the visible region whereas the controls: polymer in water (polymer without dye encapsulated) and DiI in water have no absorbance at the same region; b) dynamic light scattering; c) transmission electron microscopy; Scale bar: 500 nm

Figure 2.

a) Absorption spectra of polymer treated with different amounts of DTT to monitor the generation of pyridothione which is a byproduct during nanogel synthesis that shows characteristic absorption at 343 nm wavelength; b) Size distribution of nanogels in water as characterized by dynamic light scattering; c) TEM images of nanogels; Scale bar: 500 nm

Figure 3. DiI release from nanogels in response to 10 mM GSH over time as seen by (a) reduction in absorbance intensity over the course of 48 hours; (b) % release plot of the same

Figure 4.

Top and middle: *In vitro* cell viability of nanogels on different cell lines after 24 hours of incubation and *bottom:* confocal microscopy images of HeLa cells after incubation for 6 hours with FITC conjugated nanogels (left to right: DAPI channel, FITC channel, overlap of both channels, DIC image with overlap); Scale bar: 20 μm

Figure 5. Blastocyst development rate monitored at different pre-implantation stages: 65 of 73 zygotes in KSOM formed blastocysts and 64 of 72 zygotes in KSOM+NG formed blastocysts. Scale bar: 50 μ m.

Figure 6.

Mouse blastocyst formation and embryo transfer results. Blastocyst formation after 4 days of culture was not different with (B, B') or without (A, A') FITC-nanogels. For embryo transfer experiment, morulae/early blastocysts after 3 days of *in vitro* culture with (D, D') or without (C, C') FITC-nanogels were transferred to the uteri of pseudopregnant recipient females. Live pups born following nanogel exposure (D'') or without (C'') during preimplantation development. Scale bars: 50 μ m.

Scheme 1.

Top: Schematic representation of hydrophobic guest encapsulation, followed by redox responsive release and degradation by enzymes; *Bottom:* Chemical structure of the nanogel precursor polymer.

Scheme 2.

Synthetic scheme for the nanogel precursor polymer and nanogel.