Development of Excipient-Free Freeze-Dryable Unimolecular Hyperstar Polymers for Efficient siRNA Silencing

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Supporting Information

ABSTRACT: We designed a unimolecular hyperstar polymer for efficient small interfering RNA (siRNA) delivery that can be processed under repeated lyophilization and reconstitution without the need of any excipient. The hyperstar polymer contains a biodegradable hyperbranched core and is bound to siRNA through its thousands of cationic arms that radiate from its core. The siRNA/hyperstar complexes showed siRNA transfection efficiency that was superior to that of Lipofectamine2000 in regard to the gene for human Cu, Zn superoxide dismutase 1 (SOD1), whose mutation causes familial amyotrophic lateral sclerosis. More importantly, hyperstar polymers as unimolecular containers minimized the multipolymer cross-interaction during lyophilization, and this maintained the uniquely high transfection efficiency of the siRNA/hyperstar complexes after repeated freeze-drying and reconstitution without the conventional need for excipient.

The recent Ice Bucket Challenge media phenomenon has brought significant awareness to the research and treatment of Lou Gehrig’s disease, or ALS. Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease marked by the progressive degeneration of motor neurons, often leading to paralysis and death within 5 years of its diagnosis. Affecting approximately 6 out of every 100000 people globally, ALS currently has no cure and only one drug (Riluzole, marketed by Sanofi Pharmaceuticals and Martindale Pharma) is FDA-approved for palliative treatment. Further characterization of ALS can be made based on its origin; either sporadic (sALS) or familial (fALS). Sporadic ALS refers to the seemingly random development of the disease, as the risk factors are not clearly identified. Familial ALS is genetically induced, and can be inherited. An estimated 20% of fALS cases are directly linked to a mutation in the Superoxide Dismutase1 (SOD1) gene. Misfolded SOD1 has been found in the spinal cords of fALS patients, implicating SOD1 in the disease. Therefore, a promising therapeutic approach for fALS therapy is the targeting of mutant SOD1 in order to reduce its transcription in motor neurons.

RNA interference (RNAi) is a gene regulation mechanism in eukaryotes that can be employed as a powerful technology for sequence-specific gene silencing. In the RNAi pathway, a double stranded RNA molecule complementary to the mRNA of interest incorporates into the RNA-induced silencing complex in order to recognize the mRNA and degrade it. In this way, RNAi provides a promising technique for the silencing of mutant SOD1 toward the treatment of fALS patients with small interfering RNA (siRNA). Nevertheless, without the assistance of delivery carriers, these siRNAs treatments have remained challenging.

Due to their biocompatibility, organic biodegradable materials have been extensively explored for delivery of siRNA. Most studies focus on the use of cationic lipids and linear polymers to form self-assemblies with siRNA, termed lipoplexes and polyplexes, respectively. To date, several of these cationic complexes have been reported and demonstrate reasonable transfection efficiency. Yet, these structures are poorly defined aggregates composed of numerous randomly assembled cationic chains and anionic siRNAs that exhibit limitations on batch-to-batch product variation, non-lyophilizability, and poor stability in aqueous suspension. In particular, the poor dispersibility after lyophilization requires freshly prepared cationic complexes before administration, and this significantly limits their shelf life and stability during transport. This problem is even more pressing in developing countries where medical supplies must be transported and stored under varying conditions. Thus, additional stabilizers/excipients in practice are often added in the formulation together with the polyplexes of siRNA to achieve the product’s

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lyophilizability and maintain the siRNA-silencing efficiency.\textsuperscript{28,29} Clearly, there is a need for siRNA delivery agents with better delivery capability and further investigations into storage stability in order to combat ALS disease in clinical practice.

Here, we envision a promising siRNA carrier with a freeze-dryable format in storage and an easily reconstituted ability when used. These properties would be fulfilled by a unimolecular polymer carrier that can encapsulate siRNAs into an individual polymer without multimolecular aggregation: the development of a biodegradable hyperstar polymer (HSP) with a core–shell structure to construct a novel siRNA-delivery system for the silencing of SOD1. The core in the HSP was a hyperbranched polymer that contains degradable ester groups in polymer backbone and was synthesized using a one-pot atom transfer radical polymerization (ATRP) of AB* inimers (Figure 1, containing initiator fragment B* and polymerizable vinyl group A in one molecule) in microemulsion using conditions: $[\text{AB}^*]_0/[\text{CuBr}_2]_0/[\text{dNbpy}]_0/[$sodium ascorbate]$_0 = 70/1/2/0.5$. After the formation of the transparent microemulsion, the ATRP was initiated by an in situ reduction of Cu(II) to Cu(I) species by sodium ascorbate. The polymerization of inimer in the confined micellar space exhibited a fast polymerization rate and was stopped after an hour by exposure to air when reaching complete conversion.\textsuperscript{30,31} After polymerization, the microemulsion polymerization produced one hyperbranched polymer molecule (HB1) per micelle, which showed a monomodal peak in acetone with $D_h = 33$ nm, determined by dynamic light scattering (DLS, Figure 2C).

\begin{figure}
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\includegraphics[width=\textwidth]{image1}
\caption{Schematic of synthetic route and siRNA delivery of the polymers.}
\end{figure}

\begin{figure}
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\includegraphics[width=\textwidth]{image2}
\caption{(A) SEC curves of hyperbranched core (HB1) and the hyperstar polymer (HSP) in THF and DMF, respectively. (B) TEM image of HSP. (C) Hydrodynamic size distributions of HB1 and HSP in acetone, HSP in DI water and HSP/siRNA complexes in buffer at an N/P molar ratio (the ratio of moles of amine groups in cationic polymer to moles of phosphate groups in siRNA) of 21.79. (D) Cytotoxicity of HSP and Lipofectamine 2000 (Lipo) at different concentrations in HeLa cells (after 4 and 24 h incubation).}
\end{figure}
Further analysis of HB1 using THF size exclusion chromatography (SEC) indicated that HB1 had an apparent molecular weight, based on linear poly(methyl methacrylate) (PMMA) standards, $M_{n,PMMA} = 140 \times 10^3$ with $M_r/M_n = 1.24$. Meanwhile, the absolute molecular weight determined by a multiangle laser light scattering (MALLS, $dn/dc$ (HB1) = 0.084) detector was $M_{n,MALLS} = 1470 \times 10^3$, which is 10× higher than the apparent value, indicating a highly branched polymer structure. Since each monomer unit in HB1 had a bromine initiating site, on average this hyperbranched polymer theoretically contained 5270 inimer units. It was subsequently used as a macroinitiator for the polymerization of a functional monomer $\text{N,N}$-dimethylaminomethyl methacrylate (DMAEMA, Figure 1) in order to introduce a dense shell of tertiary amine groups around the hyperbranched polymer and to produce a core–shell structured HSP.30

The hydrodynamic size of the HSP in acetone increased from $D_h = 33$ to 58 nm at 27% DMAEMA conversion with a narrow size distribution (Figure 2B,C). The apparent molecular weight of the HSP was approximately $M_{n,PMMA} = 215 \times 10^3$ based on PMMA standards in DMF SEC (Figure 2A), although the absolute molecular weight becomes too large to be determined by using a column-based chromatography technique.33 After dialysis against pH = 6.7 water, the tertiary amine groups on the hybrid polymer were partially protonated, producing a water-dispersible HSP with $D_h = 72$ nm in pH = 7 water (Figure 2C).

The presence of both cationic protonated tertiary ammonium groups and neutral tertiary amine groups in the shell of HSP is critical since the cationic nature could bind siRNAs via electrostatic interaction.34–38 On the other hand, due to their proton absorbing capabilities inside endosomes, the neutral tertiary amine groups can lead to rapid osmotic swelling and siRNA release into the cytosol (Figure 1).39

First, HSP/siRNA complexes were prepared with different ratios of siRNA molar concentration to the HSP polymer concentration in order to determine the structure with optimal transfection efficiency in Opti-MEM. These variations of the HSP/siRNA complexes were defined by their N/P ratio (the ratio of moles in amine groups in cationic polymer to the moles inophosphate groups in siRNA). First, the complexes were characterized using DLS in order to reveal a hydrodynamic diameter of ~70 nm for the HSP/siRNA complexes in the buffer (Figure 2C), indicating that the complexes were not aggregated but, in fact, were monodisperse. The size of the HSP/siRNA complexes with a N/P molar ratio of 21.79 did not change appreciably, also indicating that the HSP/siRNA maintained its uniform structure. Furthermore, the HSP exhibited negligible cytotoxicity in HeLa cells, and this was confirmed by an MTT assay after 4 and 24 h incubation periods (Figure 2D).

We then examined the efficiency of siRNA transfection in HeLa cells, which are known to express SOD1.40 We used the chemically modified human siRNA duplex, which has been shown to be significantly selective against the human SOD1 wild-type allele (SOD1WT), and to preferentially silence the expression of the human FALS-linked mutant allele (SOD1G93A).1 In our present study, the concentration of siRNA is 50 nM. These HSP/siRNA complexes at different N/P ratios ($N/P = 0.73, 2.18, 3.63, 7.26, 14.52, \text{and} 21.79$) were transfected into HeLa cells in Opti-MEM and were then cultured for 4 h in a 6-well plate. After an incubation period of 72 h in DMEM medium containing 10% fetal bovine serum (FBS), the transfection efficiency (% of SOD1 knockdown) was measured by Western blot (Figure 3). More specifically, the protein bands were visualized using Amersham ECL kit and image of the blot was taken with Fuji LAS 4000 Imager. Further analysis using ImageJ software was used to quantify band intensities. Each lane can be the measured independently for the intensity of the signal. To analyze protein knockdown, a ratio was first calculated between the protein of interest (SOD1) and the control protein ($\alpha$-tubulin) in each lane. Then the extent of SOD1 knockdown % for each experiment is normalized to the control group (without treatments; please also see the description in page S7 of Supporting Information). This was found to increase with increasing N/P molar ratios, and it eventually reached up to 95% SOD1 knockdown at a N/P ratio equal to 21.79. It is noteworthy that, at this point, HSP shows significantly higher transfection efficiencies than Lipofectamine 2000.

Further, we examined the transfection efficiency under various conditions. First, since the artificial cerebrospinal fluid (CSF) matches that of the electrolyte concentrations and has physiological compatibility to endogenous CSF, we used a CSF to simulate the electrolyte condition where the ALS occurs. Under this condition, the transfection efficiency remains high and 97% SOD1 was silenced at an N/P molar ratio of 21.79 (Figure 4). The second attempt tested the feasibility of a freeze–thaw of the HSP/siRNA complexes and the results indicated that, after lyophilizing and reconstituting, the HSP/siRNA complexes showed a transfection efficiency of 88%. In the third experiment, we replaced the transfection solution (Opti-MEM) with a culture medium containing 10% FBS and the transfection efficiency of the HSP/siRNA complexes reached 72%. This efficiency was slightly lower than that of HSP complexes in Opti-MEM, which may be due to the interaction of polymers with FBS as competing against their interactions with siRNA. However, even under this condition, the transfection efficiency was still much higher than Lipofectamine 2000, and this confirms the superior transfection efficiency of HSP.
To evaluate the broader applications of HSP for siRNA delivery, we further measured the transfection efficiency of using unmodified siRNAs (Figure 5). In a culture medium with a 10% FBS, the HSP/siRNA complexes silenced 65% of SOD1, higher than that of Lipofectamine2000/siRNA (Figure 5). It is interesting that the transfection efficiency of the HSP/siRNA complexes was 80% after lyophilization/reconstitution and remained at 79% after incubation of this reconstitution at 37 °C for 10 days. The latter result showed that HSP efficiently protected the unmodified siRNA from degradation over a week in the solution. The HSP/siRNA complexes with no additional stabilizers can be readily lyophilized and reconstituted showing no loss of transfection efficacy. These results are critical as they suggest that the long-term storage of these HSP/siRNA complexes is a viable option for siRNA treatment that targets diseases.

In summary, we successfully designed a novel hyperstar polymer (HSP) with uniform size for application in siRNA delivery. The HSP encapsulated many siRNA molecules into one unimolecular polymer carrier that is distinct from the traditional lipoplex and polyplex aggregates. The most traditional carrier (e.g., polyplexes) require self-assembly to bind with siRNA to generate reproducible commercial products. Due to their self-assembled nature, they also typically require excipients to be added for their stability. In contrast, the hyperstar polymers serve as a self-contained monodisperse nanoparticle, and thus the siRNA only needs to be absorbed to the HSP carrier to form uniform complex. The HSP showed significantly higher siRNA transfection efficiency than Lipofectamine 2000 in various media (e.g., serum-containing media) and, more importantly, in lyophilizing/reconstitution conditions. The latter is important in clinical practice, especially in field medicine, where long-term storage of biological therapies is made possible by freeze-drying. All of these properties, including low cytotoxicity, high transfection efficiency, simple siRNA/nanoparticle self-assembly over ambient conditions, and freeze-dry ability, suggest that the HSP is a promising platform for siRNA delivery to diseases such as ALS.

**REFERENCES**


