Biodegradable Nanoparticles With Sustained Release of Functional siRNA in Skin

GUNILLA B. JACOBSON,[1] EMILIO GONZALEZ-GONZALEZ,[2,3] RYAN SPITLER,[2,3,4] RAJESH SHINDE,[2] DEVIN LEAKE,[5] ROGER L. KASPAR,[4] CHRISTOPHER H. CONTAG,[2,3,6,7] RICHARD N. ZARE[1]

[1]Department of Chemistry, Stanford University, Stanford, California 94305

[2]Department of Pediatrics, Stanford School of Medicine, Stanford, California

[3]Molecular Imaging Program at Stanford, Stanford, California

[4]TransDerm, Inc., Santa Cruz, California

[5]Thermo Fisher Scientific, Dharmacon Products, Lafayette, Colorado

[6]Department of Radiology, Stanford School of Medicine, Stanford, California

[7]Department of Microbiology and Immunology, Stanford School of Medicine, Stanford, California

Received 7 December 2009; revised 2 February 2010; accepted 19 February 2010

Published online 10 May 2010 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.22147

ABSTRACT: A key challenge in developing RNAi-based therapeutics is efficient delivery of functional short interfering RNA (siRNA) to target cells. To address this need, we have used a supercritical CO_2 process to incorporate siRNA in biodegradable polymer nanoparticles (NPs) for *in vivo* sustained release. By this means we have obtained complete encapsulation of the siRNA with minimal initial burst effect from the surface of the NPs. The slow release of a fluorescently labeled siRNA mimic (siGLO Red) was observed for up to 80 days *in vivo* after intradermal injection into mouse footpads. *In vivo* gene silencing experiments were also performed, showing reduction of GFP signal in the epidermis of a reporter transgenic mouse model, which demonstrates that the siRNA retained activity following release from the polymer NPs. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:4261–4266, 2010
Keywords: drug delivery; gene silencing; nanoparticles; supercritical fluids; transdermal drug delivery; skin; biodegradable polymer

INTRODUCTION

RNA interference (RNAi) offers potential for treating a wide variety of disorders through selective silencing of disease-relevant RNAs.[1,2] Numerous approaches have been published for targeted *in vivo* delivery of siRNA,[3,4] such as conjugates, liposomes, lipoplexes, and polymer-based nanoparticles (NPs).[5,6] The use of naked (noncomplexed, unmodified) siRNA has been applied both in mouse[7–9] and human[10,11] skin, where direct injection at the target site is possible. One example is the intradermal injection of an siRNA (designated K6a 513a.12) targeting the single nucleotide mutation in keratin 6a (K6a) encoding the N171K substitution that is responsible for the rare skin disorder pachyonychia congenita (PC).[10,11] With the objective of improved delivery, while reducing the numbers of injections needed to maintain lower expression levels of the mutant keratin gene, we encapsulated the K6a 513a.12 siRNA in a biodegradable polymer for the purpose of slow, sustained release *in vivo*. In this study the biodegradable polymer poly(L-lactic acid) (L-PLA) was used together with a copolymer of L-PLA-poly(ethylene glycol) (PEG). The addition of PEG to L-PLA was intended to improve *in vivo* circulation and reduce agglomeration of the NPs in solution.[12]

Particle formation and encapsulation experiments were performed using supercritical carbon dioxide ($SC\text{-}CO_2$) as an anti-solvent. The drug/polymer mixture was dissolved in a suitable cosolvent and sprayed through a nozzle into $SC\text{-}CO_2$, at conditions where the cosolvent is miscible with the supercritical fluid (SCF). This procedure allows rapid nucleation and particle precipitation. The high compressibility of SCFs permits adjustment of their physical properties (density, viscosity, diffusivity, etc.), which permits tight control of particle size and particle size distribution making SCFs ideal as anti-solvents.[13–15]

Correspondence to: Richard N. Zare (Telephone: 650-723-3062; Fax: 650-725-0259; E-mail: zare@stanford.edu)

Journal of Pharmaceutical Sciences, Vol. 99, 4261–4266 (2010)

The mild processing conditions (40°C and 100–300 bar) is compatible with biologically active compounds, as was shown in previous studies.[16] Other advantages of using an SCF include (1) no residual organic solvents in the particles, (2) dry particles, ready for use or long-term storage, and (3) easy process scalability.

RESULTS AND DISCUSSION

The solution enhanced dispersion using supercritical fluids (SEDS) process was performed as described previously.[17] To ensure complete encapsulation and avoid any initial burst effect, the siRNA and polymer need to be solubilized in the same cosolvent. This condition presented a challenge as the L-PLA (50 kDa) and L-PLA (70 kDa)-PEG (5 kDa) used in this study are soluble in dichloromethane (DCM), whereas siRNA is not. Initial attempts to dissolve siRNA in water, diluted with MeOH followed by mixing with L-PLA in DCM to form a homogenous solution for spraying into SEDS failed to yield NPs with high encapsulation efficiency. To overcome this limitation and to neutralize the net negative charge of the siRNA, we added the cationic lipid 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) to siRNA prior to encapsulation, to form a siRNA-DOTAP ion pair complex. DOTAP is a well-known transfection agent for siRNA delivery into cells. Once the siRNA-DOTAP complex is internalized into cells the two molecules dissociate.[18–20]

The ion pair complexation was accomplished with the Bligh–Dyer method[21] in a monophase of chloroform, water, and methanol. The monophase was formed with equimolar amounts of the siRNA and DOTAP at volume ratios of 1:2.1:1 of (chloroform/methanol/water). Upon addition of equal volumes of water and DCM, the solution mixture was phase separated into an aqueous phase and an organic phase, with the siRNA–lipid complex present in the organic phase. The siRNA–lipid complex was then transferred into DCM that contained the dissolved polymer.

Naked siRNA was dissolved in 1:100 H_2O/MeOH and siRNA-DOTAP and polymer (5–10:100 weight ratio of siRNA to polymer) were dissolved in DCM. Each solution was sprayed (1 mL/min) into SC-CO_2 (140 g/min) through a nozzle (250 μm i.d.) at 40°C and 100 bar. The particle size depends on flow rates, polymer concentration, solvent selection, temperature, and pressure. Figure 1 shows scanning electron microscope (SEM) images of (a) naked siRNA and (b) L-PLA encapsulated siRNA-DOTAP (100–300 nm in diameter) after SEDS processing. Naked siRNA showed the same PSD (20–40 nm in diameter) using both SEM and DLS analysis.[16] In DLS experiments

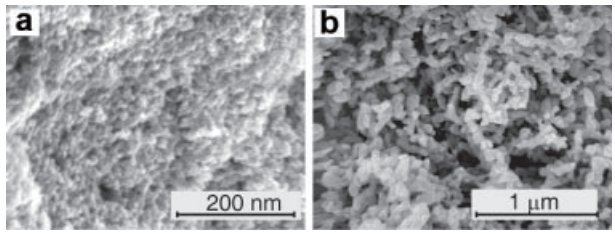

Figure 1. SEM of (a) siRNA and (b) siRNA-DOTAP encapsulated with L-PLA-PEG after SEDS processing.

the siRNA particles were dispersed in DCM and sonicated prior to measurements to prevent them from settling. L-PLA NPs tend to agglomerate and were therefore dispersed into 0.5 wt% Pluronic F68 and sonicated before DLS measurements. SEM measurements show 100–300 nm polymer particles, but DLS shows an additional peak at around 800–900 nm arising from agglomerated NPs. The size of the larger peak can be reduced by diluting the solutions and careful sonication. Addition of the PEG in L-PLA-PEG reduced the agglomeration even further and gave more uniform PSD by DLS with a PSD around 600–700 nm.

Sustained release of K6a 513a.12 siRNA from L-PLA in phosphate-buffered saline (PBS) was determined by measuring the concentration of siRNA over time, as described in the Experimental Section. The concentration of polymer in the cosolvent mixture used for SEDS affects the encapsulation efficiency, where higher concentrations are found to improve the release kinetics. No changes in particle size were observed in our experiments with the polymer concentrations used, although it may be expected that the particle size would shrink with even more dilute solutions. The total 5 wt% of siRNA to L-PLA mixed in the cosolvent was also the ratio obtained in the dry NP after SEDS, although this does not mean that all the siRNA was completely encapsulated. Figure 2a shows percent cumulative release in PBS of 5 wt% K6a 513a.12 siRNA from either a 3 or 6 mg/mL

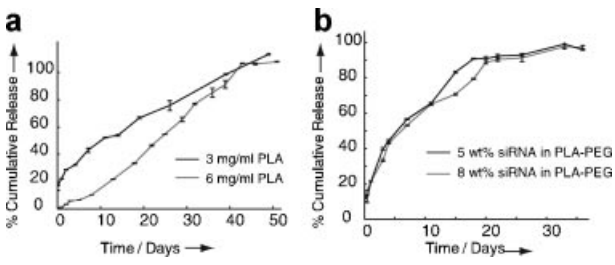

Figure 2. Percent cumulative release of siRNA in PBS from NP containing (a) 5 wt% siRNA in L-PLA (50 kDa), comparing SEDS runs using 3 or 6 mg/mL polymer concentrations in dichloromethane, and (b) 5 and 8 wt% siRNA in 6 mg/mL L-PLA (70 kDa)-PEG (5 kDa).

 DOI 10.1002/jps

L-PLA solution in DCM used for SEDS. In both cases nearly 100% recovery of siRNA was obtained, but an initial release of 15% siRNA was observed from the lower polymer concentration, caused by siRNA-DOTAP not being fully entrapped in the polymer. Using L-PLA (50 kDa), the complete release time was about 40 days.

L-PLA-PEG was also studied, which not only reduced the agglomeration of the NP, but also decreased the release time to about 20 days. Figure 2b shows percent cumulative release for a 5 and 10 wt% initial mixture of K6a 513a.12 siRNA in 6 mg/mL L-PLA-PEG in DCM. The actual amount of siRNA to L-PLA-PEG in dry NP after SEDS was determined to be 5 and 8 wt%, respectively, and the data in Figure 4 are normalized to these results. A 5 wt% loading allows for complete recovery of the siRNA after the SEDS process, while 10 wt% shows a 20% loss of siRNA in the SEDS process. All remaining experiments were therefore performed using a 5 wt% siRNA loading. As compared to using 6 mg/mL L-PLA it can be determined that L-PLA-PEG shows an initial 13% release of nonencapsulated siRNA. In all cases we are comparing dry NP as they are recovered after SEDS, without any washing steps included.

To determine the *in vivo* distribution and sustained release of siRNA from L-PLA after intradermal injection, the fluorescently labeled siRNA mimic siGLO Red was used. Intradermal injections of both 2 and 10 μg (three mice per group) naked siGLO Red and siGLO Red-DOTAP encapsulated in L-PLA were performed once in the left and right footpads of CD1 mice (Charles River, Hollister, CA), respectively. The amount of red fluorescence signal from siGLO Red siRNAs was measured by *in vivo* fluorescence imaging using an Ivis 200 imaging system as described in the Experimental Section. Figure 3 shows the decrease in fluorescence over time, where naked siGLO Red can be detected up to 4 days whereas the encapsulated siGLO Red can be monitored for up to 80 days at the higher concentration.

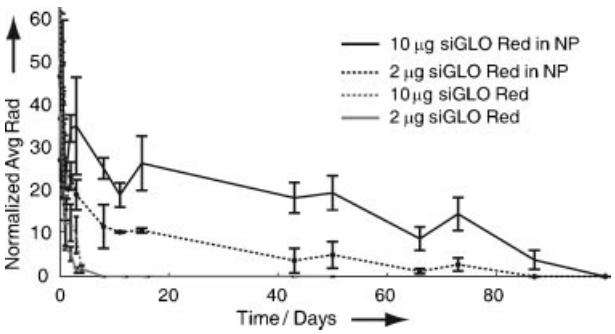

Figure 3. *In vivo* release of naked siGLO Red and siGLO Red-DOTAP encapsulated in L-PLA NPs. A total dose of either 2 or 10 μg siRNA in 50 μL PBS was injected intradermally into the footpads of CD1 mice.

To determine the gene silencing effect and thereby retained activity of the siRNA after release from L-PLA-PEG NPs, a transgenic reporter mouse model (expressing click beetle luciferase (CBL) fused to humanized Montastrea green fluorescent protein (hMGFP) in the upper layer of the epidermis) was used[7] in which CBL3 (targets the reporter gene) siRNA-mediated silencing was monitoring by measuring the decrease in epidermal hMGFP signal.

A cohort of CBL/hMGFP mice (one mouse per time point) was used to assess temporal release after injection of siGLO Red-DOTAP encapsulated in L-PLA (10 μg) in comparison to naked siGLO Red (10 μg). At 1, 4, 18, and 40 days postinjection, the footpad skin was dissected and sections were prepared to analyze the distribution of the siGLO Red in the tissue. Owing to the nature of the intradermal injection technique, most of the siGLO Red was detected in the dermis (Fig. 4), but could also be found in the epidermis (Fig. 5). The results show that the siGLO Red accumulated mostly in the dermis (blue DAPI stain identifies the nuclei) and both naked and encapsulated siGLO Red initially showed similar localization patterns (Fig. 4a,b and i,j). After 4 days, a small amount of naked siGLO Red is still present in the dermis (Fig. 4c,d), whereas the NPs show better retention both by the number of cells with red fluorescence and the signal intensity (Fig. 4k,l). Encapsulated siGLO Red NPs (but not naked siGLO Red) could be detected up to 40 days (Fig. 4o,p), which was the last time point analyzed. siGLO Red was also detected in the epidermis at the site of injection. Figure 5 shows images of siGLO Red distribution after 48 h at the site of injection of siGLO Red-DOTAP encapsulated NPs.

The CBL3 siRNA-DOTAP was encapsulated by L-PLA-PEG at 5 wt% siRNA and 6 mg/mL polymer in DCM. The encapsulation efficiency and *in vitro* release data were similar to the data shown in Figure 2b for a 5 wt% loading of K6a siRNA. Intradermal injection of 25 μg naked CBL3 siRNA or encapsulated CBL3 siRNA-DOTAP in L-PLA-PEG were performed four times over a 10-day time period in a second set of CBL/hMGFP mice. Additional injections of 25 μg CBL3 siRNA (both naked and in NP) were performed on days 4, 6, and 9. On day 10 the mice were sacrificed and tissue sections were prepared from the footpad for analysis. Figure 6 shows results from injecting naked CBL3 siRNA (Fig. 6a,b), encapsulated CBL3 siRNA-DOTAP (Fig. 6c,d), and encapsulated K6a 513a.12 siRNA-DOTAP (used as negative control; Fig. 6e,f). Both CBL3 siRNA and siRNA-DOTAP showed silencing of the hMGFP signal, that is, the expected disappearance of the green fluorescence. Note that the pictures of the silenced region do not include the complete region of gene silencing but rather were taken at the

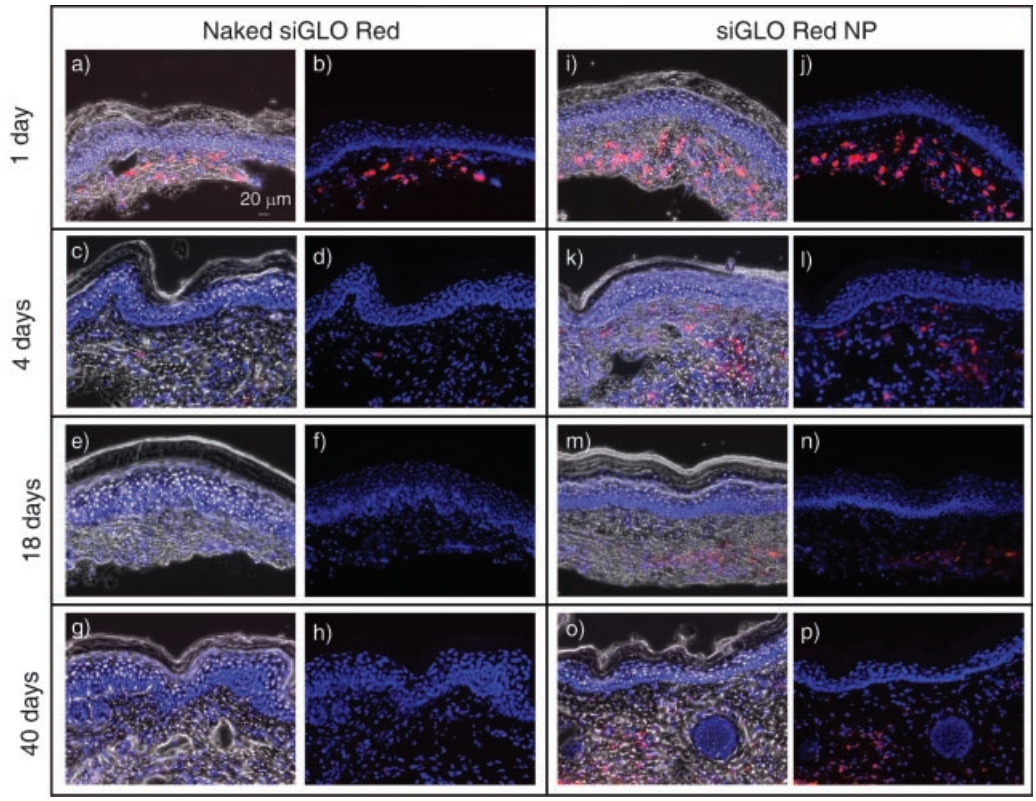

Figure 4. Images from frozen footpad skin sections of CBL/hMGFP mice (a–h) after intradermal injection of 10 μg naked siGLO Red and (i–p) after intradermal injection of 10 μg of siGLO Red-DOTAP encapsulated in L-PLA. Skin sections were obtained at 1, 4, 18, and 40 days after injection and visualized by fluorescence microscopy. Red, siGLO Red siRNA mimic; blue, DAPI-stained nuclei; left columns contain brightfield overlay.

junction of a nonsilenced area to allow direct comparison with unaffected hMGFP signal intensity in the same tissue (Fig. 6a–d). We assume that the CBL3 siRNA injections exhibit the same distribution pattern as seen in Figure 5. It should therefore be noted that the silencing effect in the epidermal region could be the result of the reservoir of siRNA accumulated there during injection. The mouse model used in this study does not allow for sustained gene silencing to be monitored, because of the constant renewal of cells in the epidermal region. These cells are regenerated over a 14-day time period as compared to our NPs which release over 20 days. We are currently working on another mouse model that will allow for silencing effect to be monitored in the dermis instead.

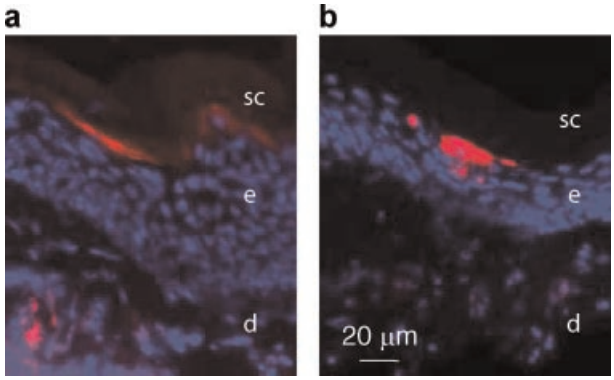

Figure 5. SiGLO Red siRNAs localized in the epidermis; (a) SiGLO Red siRNAs alone or (b) encapsulated in L-PLA nanoparticles were localized as bright deposits in the epidermis at the injection site 24 h after intradermal injection. The mechanism by which the keratinocytes take up nucleic acids remains unknown. These deposits could work as reservoirs, and siRNA could be slowly released over time. DAPI (blue) is used to stain nuclei. Scale Bar is 20 μm. Stratum corneum (sc), epidermis (e), and dermis (d).

We assume that the naked CBL3 siRNA injection site received a slightly increased total dose because a new dose was given every 3 days. We interpret these data to show that the encapsulated siRNA-DOTAP did not have sufficient time to release all its cargo at the 10-day imaging time point, and a lower total dose of siRNA was released. Nevertheless, we observe a similar knockdown level.

In this study, we have presented evidence for *in vivo* sustained release of siRNA from biodegradable NPs having high encapsulation efficiency and also shown that they remain biologically active after release. NPs composed of biodegradable polymers loaded with siRNA may prove to be an effective

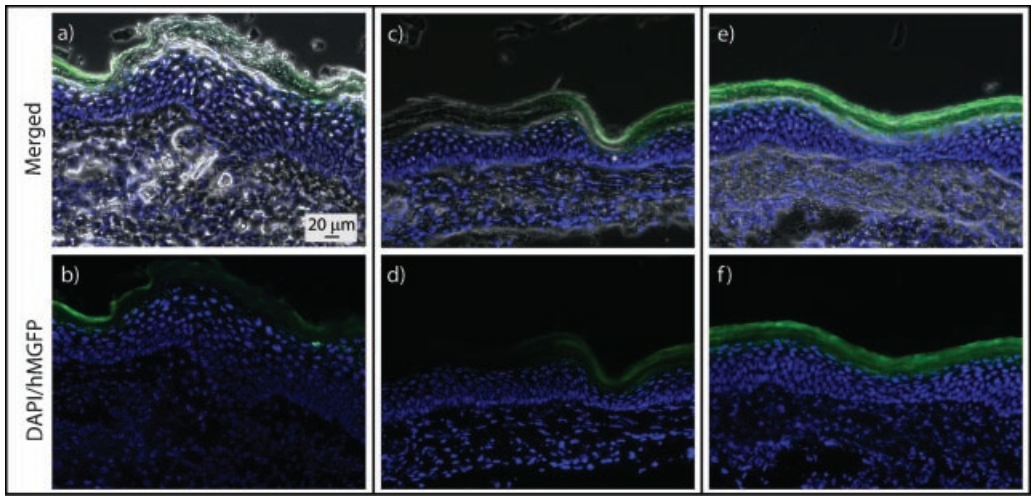

Figure 6. hMGFP silencing after intradermal injection of 25 μg CBL3 siRNA in L-PLA-PEG: (a,b) naked CBL3 siRNA (positive control), (c,d) CBL3-DOTAP encapsulated into L-PLA-PEG NP, and (e,f) K6a siRNA-DOTAP encapsulated into L-PLA-PEG NP (negative control).

therapeutic agent for controlling gene expression in the skin.

EXPERIMENTAL

Materials

Analytical grade methanol, chloroform, and DCM (Sigma–Aldrich, St. Louis, MO) were used as received. Molecular grade RNAse-free water and PBS buffer (pH 7.4) were purchased from Fisher Scientific (Pittsburgh, PA). Medical grade CO_2 with siphon tubes were purchased from Praxair (Oakland, CA). The K6a 513a.12 siRNA, CBL3 siRNA, and siGLO Red (DY-547) were provided by Thermo Fisher Scientific, Dharmacon Products (Lafayette, CO). DOTAP was purchased from Avanti Polar Lipids (Alabaster, AL), and SYBR®Gold from Invitrogen (Carlsbad, CA). L-PLA (50 kDa) was purchased from Polysciences, Inc. (Warrington, PA) and L-PLA (70 kDa)-PEG (5 kDa) was custom synthesized by Lakeshore™ Biomaterials (Bimingham, AL).

Particle Analysis

SEM images were acquired using an FEI XL30 Sirion SEM with FEG source and EDX detector. Dry samples on carbon sticky tape were sputter-coated for 45 s at 40 mA with Pd/Au. PSD were calculated on 200 well-separated particles from each SEM image and measured in zoom-in mode using Matlab.

DLS measurements were performed on a Malvern Zetasizer Nano ZS90. siRNA NPs were dispensed in DCM and sonicated for 30 s before measurements. Polymer NP were dispensed into a 0.5 wt% solution in PBS (pH 7.4) and sonicated for 30 s.

siRNA Complexation

The siRNA-DOTAP complexation was performed by adding 0.5 mg siRNA in 1 mL H_2O to 1.25 mg DOTAP in 1 mL chloroform. 2.2 mL methanol was added to form a one-phase solution and was left at room temperature for 2 h. An additional 1 mL H_2O and chloroform was added to phase separate the solution. The siRNA-DOTAP was recovered from the chloroform phase. To ensure complete conjugation, the aqueous phase was analyzed using SYBR®Gold to detect any residual siRNA. Over 98% conversion of siRNA to siRNA-DOTAP was obtained.

Release Data

Sustained release of siRNA from L-PLA or L-PLA-PEG NPs was performed by dispersing 3–5 mg particles (as received from SEDS and not washed) into 50 mL PBS solution. At each time interval 500 μL solution was removed and centrifuged for 5 min at 13.2k rpm to allow any NPs to settle. Triplicate samples of 100 μL solution was added to 50 μL SYBR®Gold (1/500 dilution) and let sit for 5 min before measuring fluorescence and comparing to a standard concentration curve.

The total amount of siRNA retained in the NP after SEDS was determined and compared to the initial mixture of either 5 or 10 wt% used in the experiments. Four to 5 mg of dry NPs were dissolved in 2 mL DCM which allowed all the polymer to dissolve. Two milliliters of PBS was then added and the siRNA

was extracted into the aqueous phase and analyzed by adding SYBR®Gold, as described above.

In Vivo Imaging

Mouse paws were imaged in an IVIS™ 200 imaging system (Xenogen product from Caliper LifeSciences, Alameda, CA) using the DsRed filter set (excitation at 460–490 nm and 500–550 nm; emissions at 575–650 nm). The resulting light emission was quantified using LivingImage software (Caliper LifeSciences), written as an overlay on Igor image analysis software (WaveMetrics, Inc., Lake Oswego, OR). DsRed background was subtracted and raw values were reported as photons per second per cm^2 and per steradian.

Tissue Preparation

Mouse sections were prepared by removing footpad skin from euthanized mice and embedded in O.C.T. (Tissue-Tek®, Torrance, CA) compound and frozen directly in dry ice. Vertical skin cross sections (10 μm) were prepared and mounted with Hydromount™ (National Diagnostic, Highland Park, NJ) containing DAPI (Sigma, St. Louis, MO) for nuclear staining.

Tissue sections were imaged with a GFP filter set (470 nm excitation, 525 nm emission) and red filter set (excitation 546 nm; emission 580 nm) in an Axio Observer Inverted Fluorescence Microscope (Zeiss, Thornwood, NY) equipped with an AxioCam MRm camera using AxioVs40 V4.6.3.0 software to visualize transgene fluorescence.

ACKNOWLEDGMENTS

This work was financially supported by the National Institutes of Health (1 R21 CA125467) (R.N.Z.) and 2R44AR055881-02 (R.L.K.), and internal grants from Stanford University as part of Bio-X IIP Program and SPARK Programs at Stanford University. EGG is the recipient of a PC Project fellowship.

REFERENCES

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391:806–811.
2. de Fougerolles A, Vornlocher H-P, Maraganore J, Lieberman J. 2007. Interfering with disease: A progress report on siRNA-based therapeutics. Nat Rev Drug Discov 6:443–453.
3. Zhang S, Zhao B, Jiang H, Wang B, Ma B. 2007. Cationic lipids and polymers mediated vectors for delivery of siRNA. J Control Release 123:1–10.
4. Novobrantseva TI, Akinc A, Borodovsky A, de Fougerolles A. 2008. Delivering silence: Advancements in developing siRNA therapeutics. Curr Opin Drug Discov Dev 11:217–224.
5. de Fougerolles AR. 2008. Delivery vehicles for small interfering RNA in vivo. Hum Gene Ther 19:125–132.
6. Whitehead KA, Langer R, Anderson DG. 2009. Knocking down barriers: Advances in siRNA delivery. Nat Rev Drug Discov 8: 129–138.
7. Gonzalez-Gonzalez E, Ra H, Hickerson RP, Piyawattanametha W, Mandella MJ, Kino GS, Leake D, Avillion AA, Solgaard O, Doyle TC, Contag CH. 2009. siRNA silencing of keratinocyte-specific GFP expression in a transgenic mouse skin model. Gene Ther 8:963–972.
8. Hickerson RP, Frances JD, Reeves RE, Contag CH, Leake D, Leachman SA, Milstone LM, McLean WHI, Kaspar RL. 2007. Single-nucleotide-specific siRNA targeting in a dominant-negative skin model. J Invest Dermatol 128:594–605.
9. Hickerson RP, Vlassov AV, Wang Q, Leake D, Ilves H, Gonzalez-Gonzalez E, Contag CH, Johnston BH, Kaspar RL. 2008. Stability study of unmodified siRNA and relevence to clinical use. Oligonucleotides 18:345–354.
10. Leachman SA, Hickerson RP, Hull PR, Smith FJD, Milstone LM, Lane EB, Bale SJ, Roop DR, McLean WHI, Kaspar RL. 2008. Therapeutic siRNAs for dominant genetic skin disorder including pachyonychia congenita. J Dermatol Sci 51:151–157.
11. Leachman SA, Hickerson RP, Schwartz ME, Bullough EE, Hutcherson SL, Boucher KM, Hansen CD, Eliason MJ, Srivatsa GS, Kornbrust DJ, Smith FJD, McLean WHI, Milstone LM, Kaspar RL. 2010. First-in-human mutation-targeted siRNA phase Ib trial of an inherited skin disorder. Mol Ther 18:442–446.
12. Alexis F, Pridgen E, Molnar LK, Farokhad OC. 2008. Factors affecting the clearance and biodistribution of polymeric nanoparticles. Mol Pharm 5:505–515.
13. Jung J, Perrut M. 2001. Particle design using supercritical fluids. J Supercrit Fluids 20:179–219.
14. Knez Z, Weidner E. 2003. Particles formation and particle design using supercritical fluids. Curr Opin Solid State Mater Sci 7:353–361.
15. Gupta RB, Chattopadhyay P. 2002. Method of Forming Nanoparticles and Microparticles of Controllable Size Using Supercritical Fluids and Ultrasound. US Patent No. 20020000681.
16. Jacobson GB, Shinde R, McCullough RE, Chen NJ, Creasman A, Beyene A, Hickerson RP, Quan C, Turner C, Kaspar RL, Contag CH, Zare RN. 2009. Nanoparticle formation of organic compounds with retained biological activity. J Pharm Sci 99: 2750–2755.
17. Jacobson GB, Shinde R, Contag CH, Zare RN. 2008. Sustained release of drugs dispersed in polymer nanoparticles. Angew Chem Int Ed 47:7880–7882.
18. Ma Z, Li J, He F, Wilson A, Pitt B, Li S. 2005. Cationic lipids enhance siRNA-mediated interferon response in mice. Biochem Biophys Res Commun 330:755–759.
19. Sioud M, Sørensen DR. 2003. Cationic liposome-mediated delivery of siRNA in adult mice. Biochem Biophys Res Commun 312:1220–1225.
20. Sørensen DR, Leirdal M, Sioud M. 2003. Gene silencing by systemic delivery of synthetic siRNA in adult mice. J Mol Biol 327:761–766.
21. Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917.