Nanoparticle Formation of Organic Compounds With Retained Biological Activity

GUNILLA B. JACOBSON,[1] RAJESH SHINDE,[2] REBECCA L. McCULLOUGH,[1] NICHOLAS J. CHENG,[1] ADAM CREASMAN,[2] AMANUEL BEYENE,[1] ROBYN P. HICKERSON,[3] CAN QUAN,[4] CHARLOTTA TURNER,[4] ROGER L. KASPAR,[3] CHRISTOPHER H. CONTAG,[2] RICHARD N. ZARE[1]

[1]Department of Chemistry, Stanford University, 333 Campus Drive, Stanford, California 94305-5080

[2]Departments of Pediatric, Radiology, Microbiology and Immunology, Stanford University School of Medicine, 318 Campus Drive, Stanford, California 94305-5439

[3]TransDerm Inc., 2161 Delaware Ave. Suite D, Santa Cruz, California 95060

[4]Department of Physical and Analytical Chemistry, Uppsala University, P. O. Box 599, SE-751 24 Uppsala, Sweden

Received 23 April 2009; revised 30 October 2009; accepted 3 November 2009

Published online 28 December 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jps.22035

ABSTRACT: Many pharmaceuticals are formulated as powders to aid drug delivery. A major problem is how to produce powders having high purity, controlled morphology, and retained bioactivity. We demonstrate the use of supercritical carbon dioxide as an antisolvent for meeting this need for two model drug systems, quercetin, a sparingly soluble antioxidant, and short interfering RNA (siRNA), which can silence genes. In both cases we achieve retention of bioactivity as well as a narrow particle size distribution in which the particles are free of impurities.
 J Pharm Sci 99:2750–2755, 2010
Keywords: siRNA; nanoparticles; supercritical fluids; particle size; biomaterials; stability; antioxidant

INTRODUCTION

Many drugs exhibit poor solubility in water (<1 mg/mL), which leads to extremely low oral bioavailability in humans.[1] The absorption of these drugs is especially affected by particle size because the bioavailability is, in most cases, dissolution-rate controlled. In the pharmaceutical industry several conventional techniques have been utilized for particle size reduction, such as spray drying, freeze drying, and liquid antisolvent precipitation.[2] Further micronization is then achieved by processes such as jet milling, pearl-ball milling, or high-pressure homogenization. When working with biologically active compounds many of these processing steps can cause loss of bioactivity caused by shock and heat sensitivity. Another drawback has been residual organic solvent that can be trapped inside the particles after drying, a major issue for pharmaceutical manufacturing.[3]

Supercritical fluids (SCFs) have become an alternative to both organic and aqueous solvents where it is highly desirable to avoid toxic organic solvents and/or higher temperatures.[4] Owing to the high compressibility of SCFs, the physical properties, such as density and viscosity, can be controlled by manipulating the pressure and temperature of the fluid. Further modifications are possible by judicious choice of additives in low concentration, such as acetone, water, and methanol (MeOH). The use of an SCF as a drying agent or antisolvent in nanoparticle formation has been shown to overcome most of the above-mentioned challenges in drug formulations.[5] The most commonly used SCF is carbon dioxide. It has the advantage of having an easily attained critical point (31.5°C and 75.8 bar). Supercritical carbon dioxide ($SC\text{-}CO_2$) is also nonflammable and inexpensive.[6] It leaves no toxic residue and is considered safe by the US Food and Drug Administration (FDA). The supercritical antisolvent is completely removed by pressure reduction, eliminating the need for additional post-treatment steps. Also, the high diffusivity of SCFs allows much faster diffusion into the liquid solvent and formation of supersaturation of the solute. This, in turn, allows for much smaller nanoparticles to be formed as well as control of the size distribution.[7] Moreover, $SC\text{-}CO_2$ antisolvent precipitation is highly scalable so that kilogram amounts can be readily achieved, if desired. It

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

Journal of Pharmaceutical Sciences, Vol. 99, 2750–2755 (2010)

remains to be demonstrated that this process preserves the biological activity of the compound that has been transformed into nanoparticles.

In this paper we describe the nanoparticle formation of two bioactive compounds, quercetin and siRNA, using SC-CO_2 as an antisolvent. Both compounds represent model drug systems that present challenges typically found in the preparation of numerous other drugs, wherein the solubility and/or stability limit their use. All experiments were run using the solution-enhanced dispersion in supercritical solvents (SEDS) process.[8] We also show retained activity of the compound of interest after SCF processing.

Quercetin (Fig. 1) is a flavonoid that is widely distributed in vegetables and plants.[9] This compound has been demonstrated to possess a wide array of biological effects that are considered beneficial to health, including antioxidative, free radical scavenging, and anticancer activity. However, quercetin is sparingly soluble (<0.01 mg/mL)[10] in water, which has limited its absorption upon oral administration.[11] Attempts to increase the solubility and oral adsorption has been investigated using both microemulsions[12] and lipid nanocapsules.[13] By generating nanoparticles of querctin, we can enhance the *in vivo* biodistribution of quercetin by making either a suspension of particles in solution or by further encapsulating them into a biodegradable polymer for slow release. The stability of quercetin was measured using a colorimetric radical scavenger method.[14]

RNA interference (RNAi) has rapidly become one of the most promising therapeutics for selectively targeting, in theory, any disease by silencing the messenger RNA (mRNA) of a gene.[15] One great challenge in using short interfering RNAs (siRNA) as therapeutics is their delivery to the cytoplasm of the target cell without degradation.[16] Several approaches are currently being investigated to stabilize and target siRNA to the delivery site, including conjugation, complexation, and lipid- or polymer-based nanoparticles. We are interested in using SCF processing for these types of siRNA-formulations and therefore investigated the stability of siRNA to SC-CO_2 processing. The functional activity of siRNA was measured using an *in vitro* transfection assay using reporter bioluminescence for rapid readout.[17]

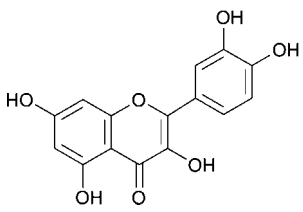

Figure 1. Molecular structure of quercetin.

EXPERIMENTAL

Materials

1,1-Diphenyl-2-picrylhydrazyl (DPPH), quercetine dihydrate, MeOH, isopropanol (IPA), ethyl acetate, dimethyl sulfoxide, and acetone were purchased from Sigma–Aldrich (St. Louis, MO) and used as received. All solvents were of analytical grade. Molecular grade RNAse-free water was purchased from Fisher (Pittsburgh, PA). Medical-grade CO_2 with siphon tubes was purchased from PraxAir (Oakland, CA). The K6a 513a.12 siRNA was supplied by Dharmacon (Lafayette, CO) Products, Thermo Scientific, Lafayette, CO. D-Luciferin was purchased from Biosynth (Itasca, IL) International.

SEDS

The SEDS equipment was based on a modified instrument (SAS50, Thar Technologies (Pittsburgh, PA)) originally set up for running the supercritical antisolvent (SAS) process. The CO_2 inlet tubing to the particle vessel has been reduced to a 1/16″ tubing with 250 μm i.d. and connected to a tee to allow premixing with the cosolvent before exiting the nozzle into the particle vessel (see Fig. 2). The system was initially conditioned by running CO_2 at the desired temperature, pressure, and flow rate. The injection pump was then started and allowing for the premixed quercetin or siRNA solution to be injected into the particle vessel. After turning off the injection pump the precipitated particles were additionally dried by continuing to flow CO_2 for an additional 30 min. After depressurizing the system the nanoparticles were scraped from the filter.

Particle Analysis

The process yield for both quercetin and siRNA was calculated in weight percent, based on the amount of each compound injected into the SEDS and how much

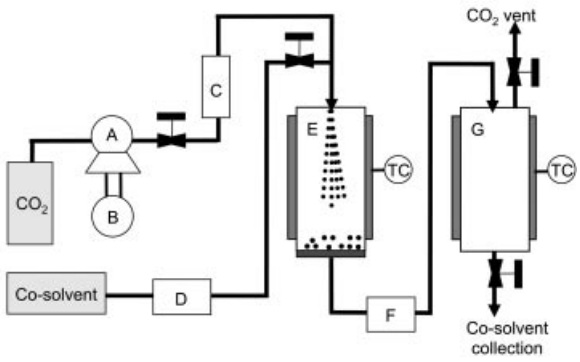

Figure 2. Schematic of the SEDS setup: A, CO_2 pump; B, cooling bath; C, heat exchanger; D, cosolvent pump; E, particle vessel; F, automatic back-pressure regulator; G, coalescer; TC, temperature controller.

was collected on the filter after the SEDS process. For the smaller batches run in these experiments (10 mg for siRNA, 20–200 mg for quercetin) it is more difficult to collect all the NPs off the filter, and therefore this affects the yield. This is especially true for siRNA because only 10 mg was injected in each experiment owing to its high cost.

Scanning electron microscope (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. Dynamic light scattering (DLS) measurements were performed on a Malvern Zetasizer Nano ZS90. PSD for siRNA was determined by both SEM and DLS (dispersed in DCM and sonicated for 30 s). PSD of quercetin samples were only determined by SEM due to the needle like structure which are not suitable for accurate DLS analysis. In this case, 200 well-separated particles from each SEM image were measured in zoom-in mode using Matlab, and the longest distance observed across each particle was taken as the particle diameter for simplicity. Particle sizes were calculated based on the ratio of their diameters to the SEM magnification scale in Matlab.

Free Radical Scavenging Method

The antioxidant activity of quercetin was estimated according to the procedure described by Brand-Williams et al.[14] A 100 μL aliquot of quercetin in ethyl acetate solution was added to 3.9 mL of a 60 μmol/L DPPH in ethyl acetate solution (made fresh daily). The reaction was found to plateau after 4 h and this time was used to measure the decrease in DPPH absorbance at 517 nm on a UNICAM 5625UV/VIS spectrometer. Aluminum foil was used to protect the samples against the possible photochemical degradation. The DPPH concentration in the reaction medium was calculated from a DPPH ethyl acetate calibration curve.

Luciferase Reporter Assay

siRNA activity was measured by cotransfection with a bioluminescent reporter expression plasmid as previously described.[17,18] Briefly, human 293FT embryonic kidney cells (Invitrogen (Carlsbad, CA)), were maintained in DMEM (CAMBREX/BioWhittaker, Walkersville, MD) with 10% fetal bovine serum (HyClone, Logan, UT), supplemented with 2 mM L-glutamine and 1 mM sodium pyruvate. The day prior to transfection, 293FT cells were seeded at 8×10^4 cells in 500 μL/well in a 48-well plate resulting in 80% cell confluency at the time of transfection. Expression plasmid (40 ng) was cotransfected with 0.25–4 μM siRNA (as received or SEDS processed) using Lipofectamine 2000 according to the manufacture's instructions. To measure firefly luciferase expression, 48 h following transfection, 50 mL of 3 mg/mL luciferin (in PBS) was added to each well and incubated at room temperature for 3–5 min. The entire plate was imaged for 20 s using the IVIS^{TM}50 *in vivo* imaging system (a Xenogen product from Caliper Life Sciences, Alameda, CA).

RESULTS AND DISCUSSION

All particle formation experiments were run using the SEDS process, which is illustrated in Figure 2. Several process parameters were investigated to study the effect on both particle size and morphology of quercetin and siRNA, using SEM imaging and DLS methods. Decreased particle size was found by optimizing several process parameters for each choice of compound and cosolvent mixture, as described below.

Quercetin

The choice of cosolvent is based on the solubility of the compound of interest as well as its compatibility with SC-CO_2. The cosolvent must initially solubilize the compound, and as it is sprayed into the particle vessel the process conditions must allow for complete solubility with SC-CO_2, allowing for the precipitation of the compound, which is not soluble in SC-CO_2 (a prerequisite for using SEDS). There are several options of cosolvents for quercetin, as it is highly soluble in many organic solvents that are miscible with SC-CO_2. We investigated the use of dimethyl sulfoxide (DMSO), MeOH, IPA, acetone, ethyl acetate, and acetonitrile.

The process temperature and pressure was chosen to allow for complete miscibility of the cosolvent with CO_2. A variable volume view cell was used to determine the phase diagram of each cosolvent mixture to ensure a supercritical phase in the process.[19] The effect of temperature and pressure parameter variation on yield and particle size was determined in a set of SEDS process experiments; while keeping constant the CO_2 flow rate (150 g/min), cosolvent injection rate (1 mL/min), and quercetin concentration (0.5 mg/mL), the temperature and pressure were varied from 40, 60, and 80°C and 90, 100, 200, and 300 bar. For all cosolvents studied, a higher pressure gave the smaller particle size as well as increased yield. In each case, the effect of temperature was not significant.

Increasing the CO_2 flow rate and decreasing the injection flow rate will increase the ratio of SC-CO_2 to cosolvent at the point of injection, allowing for smaller particle size. The CO_2 flow rate was varied from 50 to 150 g/min and the injection rate from 1 to 5 mL/min. These are the limits of the experimental SEDS equipment used. The optimal process conditions for our equipment were found to be 150 g/min

of CO_2 and 1 mL/min injection rate, that is, at the highest attainable ratio of CO_2 to cosolvent. The concentration of quercetin dissolved in cosolvent was varied from 0.2 to 2.0 mg/mL, with the smaller particles resulting from the lower concentrations. The yield on the other hand increased with increasing quercetin concentration.

With all cosolvents studied at any given process condition, the morphology of quercetin particles was needle-like. The choice of cosolvent did affect the yield and size of the particles, MeOH and IPA yielding the smallest particles (10–30 nm wide, 1–10 μm long) and the others larger, thick, rod-like particles. MeOH and IPA also gave higher yields, in the range of 75–80% as compared to the other solvents where the yields ranged from 50% to 70%. This behavior is explained by the decreased solubility of quercetin in the alcohols, allowing for faster supersaturation when mixed with SC-CO_2. Figure 3 shows an example of SEM images of quercetin before and after the SEDS process.

The antioxidant activity of quercetin before and after SEDS was estimated according to the procedure described by Brand-Williams et al.[14] DPPH is a stable free radial with maximum absorbance at 517 nm, and is commonly used to measure antioxidant activity of compounds.[20] Figure 4 shows the percentage of residual DPPH at steady state (after 4 h reaction at room temperature) as a function of the molar ratio of quercetin to DPPH for quercetin before and after SEDS, respectively. The percentage of remaining DPPH (% $DPPH_{REM}$) was calculated from

$$DPPH_{REM}(\%) = [DPPH]_T/[DPPH]_{T=0} \times 100$$

The EC_{50} value is extrapolated from each plot, which is the amount of quercetin needed to decrease the absorbance of DPPH by 50%. The EC_{50} value for unprocessed (0.1436) and processed (0.1392) quercetin particles are within the same range. The slightly decreased EC_{50} value after SEDS processing is likely caused by extraction of impurities from the unprocessed quercetin. The results show that the SC-CO_2 antisolvent precipitation process retains the antioxidative activity of quercetin.

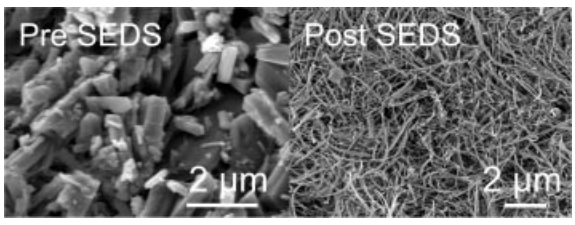

Figure 3. SEM image of quercetin before and after SEDS. Process conditions: 40°C, 100 bar, 140 g/min CO_2, 1 mL/min injection flow, 1 mg/mL quercetin in methanol.

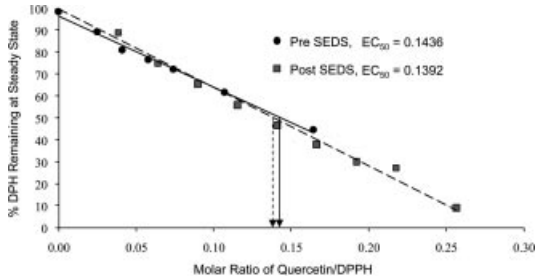

Figure 4. DPPH data showing EC_{50} of quercetin before and after SEDS.

siRNA

The siRNA (K6a 513a.12) used in this study was a 21 nucleotide, double-stranded siRNA targeting the single-nucleotide keratin 6a (K6a) N171K mutation responsible for the rare skin disorder pachyonychia congenita (PC).[17] PC is caused by mutations in one of the inducible keratin genes; however, most patients carry K6a mutations. Previous studies of this siRNA targeting the K6a gene[17] allowed us to use it as a positive reference to its compatibility with our SEDS process. This siRNA is the non-GMP version of TD101 siRNA, which was recently used in a Phase 1b clinical trial for PC.[21]

siRNA belongs to a broad class of water-soluble compounds with low or insignificant solubility in most organic solvents. This presents a challenge when choosing a cosolvent for the SEDS process, because pure water has extremely low solubility in SC-CO_2.[22] siRNA was solubilized in a small amount of water and diluted 1:100 with MeOH to increase the solubility of the water in SC-CO_2. The temperature and pressure were varied between 40–80°C and 100–300 bar. The trend in particle size and yield was mostly the same as for quercetin, with higher CO_2 flow rate, lower injection flow rate, and higher pressure yielding smaller particles. The temperature on the other hand had a markedly greater affect on the siRNA process, with an increase in yield from 15% to 58% when increasing the temperature from 40 to 60°C. This behavior is most likely explained by the increased solubility of water in SC-CO_2 at higher temperatures. Figure 5 shows SEM images of siRNA after SEDS,

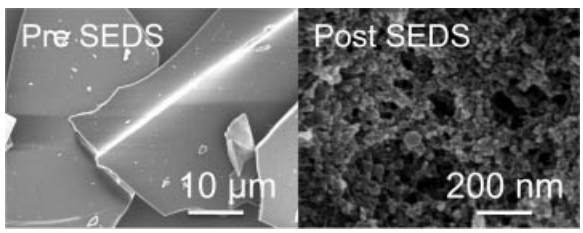

Figure 5. SEM image of siRNA before and after SEDS. Process conditions: 60°C, 100 bar, 150 g/min CO_2, 1 mL/min injection flow, 0.5 mg/mL siRNA in 100 μL water, diluted with 10 mL methanol.

run at 150 g/min, 1 mL/min injection, 60°C, 100 bar and an siRNA concentration of 0.5 mg/mL in water. The particle size ranged from 20 to 40 nm as determined by both SEM and DLS.

The activity of the siRNA before and after the SEDS process (100 bar, 80°C, 150 g/min CO_2, 1 mL/min 1:100 H_2O/MeOH, 10 mg siRNA in 100 μL H_20 and 10 mL MeOH) was tested using a functional activity assay. The mutant and wild-type K6a coding regions were individually linked to the firefly luciferase (fLuc) gene as a reporter to rapidly reveal activity of siRNA with a rapid assay using bioluminesence.[17] This assay allows the activity of the siRNA to be measured by observing a decrease in the reporter bioluminescent signal upon transfection with increasing amounts of siRNA. K6a 513a.12 targets the K6a N171K single nucleotide mutation but does not affect wild-type expression.[16] Figure 6 shows the results of the *in vitro* assay, where the wild-type gene is used as the control. It can be seen that the siRNA both before and after SEDS shows the same activity. NPs made by SEDS at both 40 and 80°C have been tested with this assay, all showing retained biological activity. The pure K6a siRNA was also heated to 80°C and tested to show the stability of the siRNA at these temperatures using the same assay.

siRNA NPs alone are not suitable for direct *in vivo* delivery due to water solubility in the aqueous buffer phase, which would be used for IV injection, as well as rapid degradation *in vivo* by macrophages before reaching the target site. These active siRNA nanoparticles can now be embedded into biodegradable nanopolymers using the same SEDS process, which is work currently in progress in our laboratory. The MW and chemical structure of the polymers can be optimized to attain the desired release time, and the attachment of targeting moieties on the NP surface will allow for more targeted delivery to specific sites.[23]

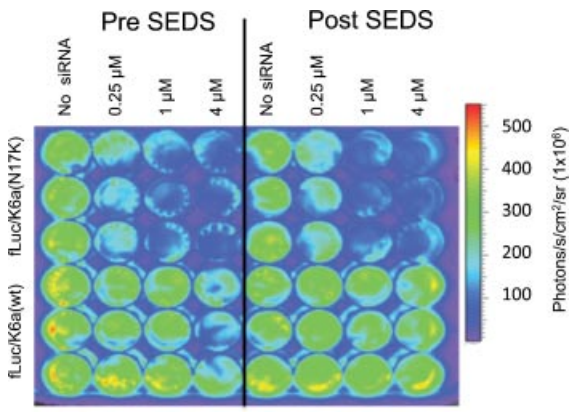

Figure 6. *In vitro* assay showing functional activity of siRNA after SEDS as compared to unprocessed. siRNA targets only the mutant K6a mRNA and not wild-type expression shown below as control. 293FT cells were cotransfected in triplicate with the indicated amounts of unprocessed (pre-SEDS) or processed (post-SEDS) siRNAs and wild-type or mutant (N171K) fLuc/K6a expression constructs in a 48-well plate format. After transfection (48 h), luciferin substrate was added and light emitted was visualized using the Xenogen IVIS™50 *in vivo* imaging system.

CONCLUSIONS

Nanoparticles of the antioxidant quercetin and the siRNA (K6a 513.a.12) were formed by injecting solutions of these two compounds into supercritical carbon dioxide, which acts as an antisolvent to cause precipitation. The particle size and particle size distribution could be controlled by optimizing mainly the flow rates, temperature, and pressure, as well as the cosolvent choice and concentration of drug in the cosolvent. Most importantly, bioassays performed on the nanoparticles showed that they retained their activity under these rather mild processing conditions. We suggest that this behavior is general, which opens the possibility of processing both hydrophilic and hydrophobic pharmaceuticals into nanoparticles with maintained desired activity in a wide number of instances. We are currently in the process of embedding these nanoparticles in biodegradable polymers for sustained release or to use them directly, either by aerosolization of inhalation or by putting them into creams for transdermal delivery.

ACKNOWLEDGMENTS

The authors thank the National Institutes of Health (1 R21 CA125467 and R43ARO53789 (RLK)), the Bio-X IIP Program at Stanford and VINNOVA (The Swedish Governmental Agency for Innovation Systems) for financial support. C. Turner acknowledges the Swedish Research Council (VR, 2006-4084) and the Swedish Foundation for Strategic Research (SSF, 2005:0073/13).

REFERENCES

1. Kesisoglou F, Panmai S, Wu Y. 2007. Nanosizing—Oral formulation development and biopharmaceutical evaluation. Adv Drug Deliv Rev 59: 631–644.
2. Rasenack N, Muller BW. 2004. Micron-size drug particles: Common and novel micronization techniques. Pharm Dev Technol 9:1–13.
3. Giry K, Péan JM. 2006. The influence of carrier and drug morphology on drug delivery from dry powder formulations. Int J Pharm 321:162–166.
4. 4aXu CY, Sievers RE, Karst U, Watkins BA, Karbiwnyk CM, Andersen WC, Schaefer JD, Stoldt CR. 1998. Supercritical carbon dioxide assisted aerosolization for thin film deposition,

fine powder generation, and drug delivery. In: Anastas PT, Williamson TC, editors. Green chemistry: Frontiers in benign chemical synthesis and processing. Oxford: Oxford University Press. pp. 312–334;4.;
4bHuang ETS, Chang H-Y, Liang CD, Sievers RE. 2003. Fine particle pharmaceutical manufacturing using dense carbon dioxide mixed with aqueous or alcoholic solutions. ACS Symp Ser 860:324–338.
5. 5aJung J, Perrut M. 2003. Particle design using supercritical fluids. J Supercrit Fluids 20:179–219;5.;
5bKnez Z, Weidner E. 2003. Particles formation and particle design using supercritical fluids. Curr Opin Solid State Mater Sci 7:353–361.
6. Ikariya T, Noyori R, Abrams MB, Carter CAG, Jacobson GB, Liu FC, Pesiri DR, Tumas W. 2000. Advances in homogeneous heterogeneous and biphasic metal-catalyzed reactions in dense-phase carbon dioxide. In: DeSimone J, Tumas W, editors. Green chemistry using liquid and supercritical carbon dioxide. New York: Oxford University Press. pp. 17–47.
7. Gupta RB, Chattopadhyay P. 2002. Method of forming nanoparticles and microparticles of controllable size using supercritical fluids and ultrasound. US Patent 20020000681.
8. Hanna MH, York P. 1998. Method and apparatus for the formation of particles. US Patent 5851453.
9. 9aErlund I. 2004. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. Nutr Res 24:851–874;9.;
9bFormica JV, Regelson W. 1995. Review of the biology of quercetin and related bioflavonoids. Food Chem Toxicol 33: 1061–1080.
10. Chebil L, Humeau C, Anthoni J, Dehez F, Engasser J-M, Ghoul M. 2007. Solubility of flavonoids in organic solvents. J Chem Eng Data 52:1552–1556.
11. Zheng Y, Haworth IS, Zuo Z, Chow MSS, Chow AHL. 2005. Physicochemical and structural characterization of quercetin-beta-cyclodextrin complexes. J Pharm Sci 94:1079–1089.
12. Gao Y, Wang Y, Ma Y, Yu A, Cai F, Shao W, Zhai G. 2009. Formulation optimization and in situ absorption in rat intestinal tract of quercetin-loaded microemulsion. Colloids Surfaces B 71:306–314.
13. Barras A, Mezzetti A, Richard A, Lazzaroni S, Roux S, Melnyk P. 2009. Formulation and characterization polyphenol-loaded lipid nanocapsules. Int J Pharm 379:270–277.
14. Brand-Williams W, Cuvelier ME, Berset C. 1995. Use of a free-radical method to evaluate antioxidant activity. Food Sci Technol-LEB 28:25–30.
15. de Fougerolles A, Vornlocher H-P, Maraganore J, Lieberman J. 2007. A progress report on siRNA-based therapeutics. Nat Rev Drug Discov 6:443–453.
16. Novobrantseva TI, Akinc A, Borodovsky A, de Fougerolles A. 2008. Delivering silence: Advancements in developing siRNA therapeutics. Curr Opin Drug Discov Devel 11:217–224.
17. 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 relevance to clinical use. Oligonucleotides 18:345–354.
18. Hickerson RP, Smith FJD, Reeves RE, Contag CH, Leake D, Leachman SA, Milstone LM, Irwin McLean WH, Kaspar RL. 2008. Therapeutic siRNAs for dominant genetic skin disorder including pachyonychia congenita. J Invest Dermatol 128:594–605.
19. Jacobson GB, Lee CT Jr, Johnston KP, Tumas W. 1999. Enhanced catalyst reactivity and separation using water/carbon dioxide emulsions. J Am Chem Soc 121:11902–11903.
20. 20aBerger JM, Rana RJ, Javeed H, Javeed I, Schulien SL. 2008. Radical quenching of 1,1-diphenyl-2-picrylhydrazyl: A spectrometric determination of antioxidant behavior. J Chem Edu 85:408–410;20.;
20bSanchez-Moreno C, Larrauri JA, Saura-Calixto F. 1998. A procedure to measure the antiradical efficiency of polyphenols. J Sci Food Agric 76:270–276;20.;
20cJimenez-Escrig A, Jimenez-Jimenez I, Sanchez-Moreno C, Saura-Calixto F. 2000. Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picrylhydrazyl. J Sci Food Agric 80:1686–1690.
21. Leachman A, 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 disorders including pachyonychia congenita. J Dermatol Sci 51:151–157.
22. Wiebe R, Gaddy VL. 1941. Vapor phase composition of carbon dioxide-water mixtures at various temperatures and at pressures to 700 atmospheres. J Am Chem Soc 63:475–477.
23. Novobrantseva TI, Akinc A, Borodovsky A, de Fougerolles A. 2008. Delivering silence: Advancements in developing siRNA therapeutics. Curr Opin Drug Discov Devel 11:217–224.