Sustained release of nucleic acids from polymeric nanoparticles using microemulsion precipitation in supercritical carbon dioxide

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A general approach for producing biodegradable nanoparticles for sustained nucleic acid release is presented. The nanoparticles are produced by precipitating a water-in-oil microemulsion in supercritical CO_2. The microemulsion consists of a transfer RNA aqueous solution (water phase), dichloromethane containing poly(L-lactic acid)–poly(ethylene glycol) (oil phase), the surfactant *n*-octyl β-D-glucopyranoside, and the cosurfactant *n*-butanol.

The possibility of using nucleic acids for pharmacological purposes has gained a new impetus with the discovery of small interfering RNA (siRNA) to silence genes.[1] Despite the many advances of siRNA therapeutics a major stumbling block remains in the delivery of the siRNA, to protect the nucleic acid from degradation until it enters a cell of interest and to provide sustained delivery of the siRNA.[2] There are many approaches being investigated for targeted delivery of siRNA, including both viral and non-viral delivery vehicles. For the non-viral methods the use of chemical conjugation, or incorporation into liposomes, lipoplexes, and polymeric nanoparticles has been the most successful.[3]

Polymeric nanoparticles can be formed by precipitation of the nucleic acid and polymer into an anti-solvent. In our case, the polymer and nucleic acid are dissolved in a solvent system that is added to an anti-solvent that allows for precipitation of the polymer with incorporated drug. Most polymers are only soluble in organic solvents and therefore require the nucleic acid to be conjugated to a hydrophobic entity. The negatively charged nucleic acid cannot cross the cell membrane by itself and can be coupled to for example a cationic lipid (such as *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N* trimethylammonium methylsulfate, DOTAP) to form a hydrophobic ion-pair.[4] Many of the commonly used transfection agents form a hydrophilic complex with nucleic acids, which are used for direct injection *in vivo* in an aqueous phase. To encapsulate these conjugated nucleic acids into a polymeric nanoparticle requires a two-phase system, both aqueous and organic. Another option would be to design the nanoparticle itself to cross the cell membrane and deliver the hydrophilic nucleic acid directly into the cytoplasm.

Here we demonstrate a process using precipitation from a water-in-oil microemulsion to allow for a hydrophilic nucleic acid to be incorporated into a biodegradable polymer for sustained release *in vivo* (see Fig. 1). Transfer RNA (tRNA, Roche Diagnostics) was used as a model compound, but could simply be replaced by any active RNA or DNA complex soluble in water. The biodegradable and FDA approved copolymer poly(L-lactic acid)–poly(ethylene glycol) (PLLA–PEG, MW 70 kD–5 kD, Lakeshore Biomaterials) was used to provide sustained release.[4] Nanoparticles made from a copolymer of PLA and PEG have previously been demonstrated to be effective for DNA delivery.[5] The PEG segment has been shown to prolong nanoparticle blood circulating half-life *in vivo*, owing to the ability of PEG to reduce nonspecific protein binding and prevent opsonization and subsequent recognition by macrophages.[6]

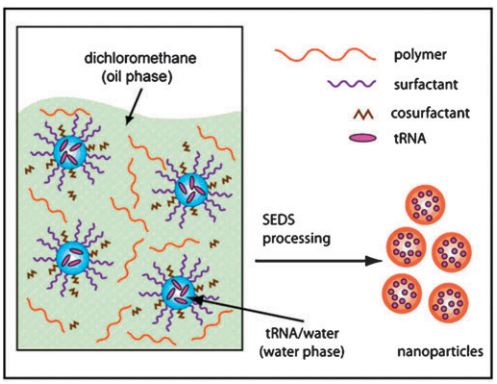

Fig. 1 Schematic representation of water-in-oil microemulsion and polymeric nanoparticles prepared by supercritical CO_2 processing. The relative size of molecular species are exaggerated for illustrative purposes.

Several studies have demonstrated that nanoparticles prepared from biodegradable polymers using supercritical carbon dioxide (SC-CO_2) as an anti-solvent can be successfully used for drug encapsulation and protection while retaining the biological activity of the nucleic acid.[7] The high compressibility of a supercritical fluid allows for control of the anti-solvent properties of the fluid and thereby solubility of the polymer and drug. SC-CO_2 is also non-toxic, non-flammable, and FDA approved. Such a process can readily be scaled to kilogram quantities, and represents a new approach with great potential to produce dry polymeric nanoparticles without any trace of organic solvents for sustained drug delivery. In this work we use the solution enhanced dispersion by supercritical fluids (SEDS) method to produce nanoparticles, where SC-CO_2 is used as an antisolvent.[8] In general, a mixture of drug and polymer is

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dissolved in a suitable solvent and sprayed through a nozzle into a SC-CO_2 stream. The high diffusivity of the supercritical fluid allows for rapid nucleation and precipitation of polymer particles with incorporated drug. The nanoparticles are collected dry after depressurizing the carbon-dioxide into a gas, thereby eliminating any solvent removal steps.

The mixture of water, a water-immiscible organic liquid, and an amphiphile generally forms a turbid milky emulsion that separates with time into an aqueous and an organic phase. This turbid unstable emulsion can be converted into an optically transparent and thermodynamically stable micro-emulsion by adding alcohol (*i.e.*, *n*-butanol) as a co-surfactant.[9] Micro-emulsions consist of oil and water domains in the nanometre-size range covered with a monolayer of surfactant. These domains constantly interact and represent a highly dynamic system. Microemulsions are similar to micellar systems, but with the added oil phase dissolved in the apolar surfactant tail region. Emulsions are very different from microemulsions in that they require mechanical energy to form, are not thermodynamically stable, and have larger micron-sized domains. They are static systems where the droplets do not interact and have considerably less surface area as compared to a microemulsion. Microemulsions have been widely used in various industrial processes such as oil recovery, extraction, pharmaceuticals, cosmetics, chemical reactions, *etc*.[10]

To encapsulate a hydrophilic nucleic acid a water-in-oil microemulsion was formed consisting of aqueous nano-droplets of nucleic acid solution, dichloromethane containing PLLA–PEG, the surfactant *n*-octyl β-D-glucopyranoside, and the cosurfactant *n*-butanol, Fig. 1. The microemulsion was injected into supercritical CO_2, causing the precipitation of polymer nanoparticles with the nucleic acid molecules incorporated inside the polymer. This process is to be distinguished from supercritical fluid extraction of emulsions (SFEE), which is based on a principle whereby nanoparticle suspensions are produced by supercritical fluid extraction of the organic solvent from an oil-in-water emulsion.[11] The present study is, to the best of our knowledge, the first report of combining microemulsions and an SC-CO_2 precipitation process to produce polymeric nanoparticles that incorporate a water-soluble drug, in our case, tRNA.

Formulating nonionic microemulsions with a chlorinated hydrocarbon as oil component is not straight-forward, simply because the good solvency of chlorinated solvents will make most nonionic surfactants go into the oil domain rather than to the interface where they are needed. Polyol-based surfactants are an exception. Their polar headgroup is very lipophobic in such systems. A sugar-based surfactant, such as *n*-octyl β-D-glucopyranoside (molecular structure shown in Fig. 2a) is therefore a natural choice. This surfactant is considered to be a biodegradable and "green" surfactant.[12] In a typical experiment, 40 mg of surfactant was dissolved in 6 mL of dichloromethane, followed by addition of 40 μL of water, forming a milky emulsion, as shown in Fig. 2b, right hand image. Under gentle stirring, 150 μL of *n*-butanol was slowly added to the emulsion, resulting in a thermally stable, homogeneous, transparent microemulsion (Fig. 2b, left hand image). The size of the water droplets in this microemulsion, as determined by dynamic light scattering, was around 15 nm

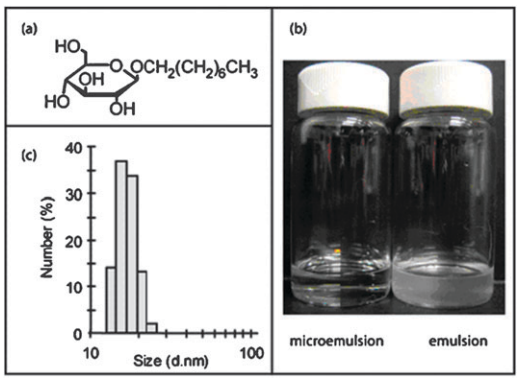

Fig. 2 Microemulsion (a) Molecular structure of *n*-octyl β-D-glucopyranoside. (b) The photograph image of microemulsion (left) and emulsion (right). (c) The size distribution of water droplets in the microemulsion as determined by dynamic light scattering.

(Fig. 2c). By varying the water content from 0.25 wt% to 1.20 wt%, the sizes of the water droplets ranged from several nanometres to around two hundred nanometres.

To incorporate tRNA into the nanoparticles we used the same microemulsion formulation as described above, but with tRNA dissolved in the water component (0.025–0.1 mg μL^{-1}), and PLLA–PEG (5 mg mL^{-1}) dissolved in the oil component. The SEDS process was used as is described in our previous studies.[7] Briefly, the microemulsion was injected (1 mL min^{-1}) through a nozzle with 250 μm internal diameter into a SC-CO_2 (150 g min^{-1}) at 40 °C and 100 bar. After collecting and weighting the dry nanoparticles, we estimate the yield of our SEDS process to be ~58%. ^{1}H-NMR (600 MHz, solvent: $CDCl_3$, δ = 5.28–5.11 (–OC-CH(CH_3)O–), 3.60 (–CH_2CH_2O–), 1.62–1.45 (–OC-$CHCH_3$O–)) showed no residual *n*-octyl beta-D-glucopyranoside in dry nanoparticles, indicating that it had been removed by the supercritical CO_2.

To measure the drug loading ratio of tRNA in dry polymeric nanoparticles (the total amount of tRNA after the SEDS processing) 5 mg of tRNA-encapsulated polymeric nanoparticles were dissolved in 5 mL of dichloromethane followed by addition of 5 mL of phosphate buffered saline (PBS, pH 7.4) to extract the tRNA into PBS. The concentration of tRNA in aqueous solution was measured by using the fluorescent dye SYBR®Gold™ (Invitrogen) and comparing to a standard concentration curve. The drug loading ratio was measured to be approximately 2.3–5.1 wt% with different initial concentrations of tRNA in the microemulsions (0.025–0.1 mg μL^{-1}). The size distribution of the nanoparticles was measured by using scanning electron microscopy (SEM) and dynamic light scattering (DLS), Fig. 3a and b. The nanoparticles have sizes around 200 to 700 nm as determined by SEM, and the DLS measurements (Malvern Zetasizer Nano ZS90) showed that the nanoparticles dispersed in a PBS solution have an average size around 680 nm due to some aggregates of nanoparticles formed in aqueous solutions. The zeta potential of tRNA encapsulated polymeric nanoparticle in PBS (pH 7.4) was measured to be −11.4 mV. The tertiary structures of native tRNA and tRNA extracted from the nanoparticles were compared by agarose gel electrophoresis (Fig. 3c). No difference in the tRNA bands

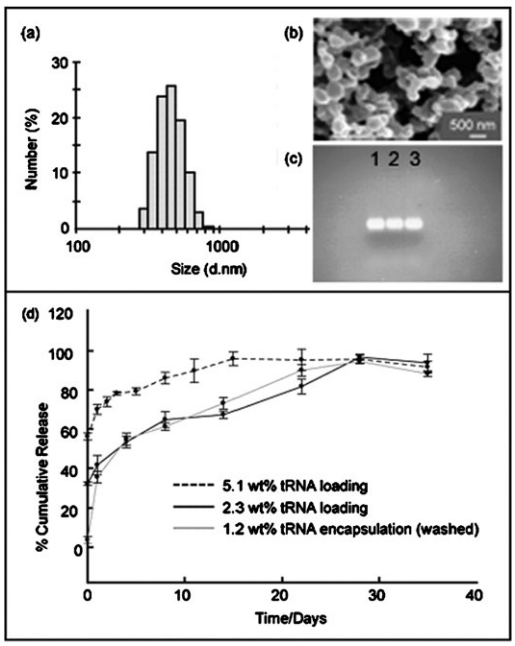

Fig. 3 Polymeric nanoparticles with incorporated tRNA. (a) Dynamic light scattering measurement of tRNA encapsulated PLLA–PEG nanoparticles dispersed in pH 7.4 PBS. (b) SEM image of PLLA–PEG nanoparticles with encapsulated tRNA. (c) Agarose gel electrophoresis, 1.2% gel, ethidium bromide stained. Native tRNA (lane 1), tRNA extracted from nanoparticles with 5.1 wt% (lane 2) and 2.3 wt% (lane 3) tRNA loading. (d) Cumulative release of tRNA from PLLA–PEG nanoparticles.

was observed, indicating that there was no degradation or denaturation of tRNA during the emulsion process and precipitation in supercritical CO_2. The preserved bioactivities of proteins and nucleic acids have been demonstrated in various emulsion-based processes[13] as well as in our previous work on *in vivo* siRNA delivery.[4b]

To monitor the sustained release of tRNA from PLLA–PEG nanoparticles, 5.0 mg of particles were dispersed in 40 mL of PBS at 37 °C. At a given time point, 500 μl of nanoparticle suspension was removed and centrifuged for 5 min at 13.2k rpm, which allowed for any solid nanoparticles to settle. The concentration of released tRNA in the aqueous component was measured by adding SYBR®Gold™, which only binds to free tRNA. Fig. 3d shows three different curves of the cumulative release of tRNA. In all three curves, the first release data point was obtained after 5 min incubation.

The nanoparticles prepared with 5.1 wt % tRNA loading (dashed curve) release about 60% of the tRNA during the first 5 minutes. Thus, only 40% of the tRNA was encapsulated inside the particle. Lowering the amount of tRNA loaded to 2.3 wt% (solid black curve) allows for encapsulating of approximately 70% of tRNA, and lowering the initial unencapsulated release to 30% tRNA. Thereafter, a slow release of tRNA over 2 to 3 weeks was observed. Note that the nanoparticles with 5.1 and 2.3 wt% loading ratio were not washed after SEDS processing.

In order to emphasize encapsulation and sustained release of tRNA, 10 mg of the particles with 2.3 wt% tRNA loading were washed with PBS and centrifuged two times to remove all unencapsulated tRNA. After the washing step, the nanoparticles were suspended in 40 mL of PBS buffer, and the sustained release of tRNA was monitored over time. The amount of tRNA encapsulated in the particles was measured to be 1.2 wt%. Fig. 3d (grey curve) shows that there is no free tRNA present during the first measurement, which indicates that all unencapsulated tRNA has been removed during the washing step. The tRNA release from the washed particles lasted for 3 weeks, which is similar to the unwashed samples.

In conclusion, we present a general method for producing biodegradable nanoparticles that can be loaded with nucleic acids. The advantages of this method include easy scalability, no residual organic solvents, and dry nanoparticles that can be used for long-term storage.[14] As a further step for increasing the capacity of nanoparticles to enter a cell and to achieve targeting of specific cell types, we suggest modifying the polymer with the targeting moieties, such as ligands, peptides or aptamers.[15]

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