Intravitreal Sustained Delivery of Rapamycin

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PURPOSE. To understand the relationship between rapamycin loading/release and surface chemistries of porous silicon (pSi) to optimize pSi-based intravitreal delivery system.

METHODS. Three types of surface chemical modifications were studied: (1) pSi-COOH, containing 10-carbon aliphatic chains with terminal carboxyl groups grafted via hydrosilylation of undecylenic acid; (2) pSi-C12, containing 12-carbon aliphatic chains grafted via hydrosilylation of 1-dodecene; and (3) pSiO2-C8, prepared by mild oxidation of the pSi particles followed by grafting of 8-hydrocarbon chains to the resulting porous silica surface via a silanization.

RESULTS. The efficiency of rapamycin loading follows the order (micrograms of drug/milligrams of carrier): pSiO2-C8 (105 ± 18) > pSi-COOH (68 ± 8) > pSi-C12 (36 ± 6). Powder X-ray diffraction data showed that loaded rapamycin was amorphous and dynamic drug-release study showed that the availability of the free drug was increased by 6-fold (compared with crystalline rapamycin) by using pSiO2-C8 formulation (P = 0.0039). Of the three formulations in this study, pSiO2-C8-RAP showed optimal performance in terms of simultaneous release of the active drug and carrier degradation, and drug-loading capacity. Released rapamycin was confirmed with the fingerprints of the mass spectrometry and biologically functional as the control of commercial crystalline rapamycin. Single intravitreal injections of 2.9 ± 0.57 mg pSiO2-C8-RAP into rabbit eyes resulted in more than 8 weeks of residence in the vitreous while maintaining clear optical media and normal histology of the retina in comparison to the controls.

CONCLUSIONS. Porous silicon–based rapamycin delivery system using the pSiO2-C8 formulation demonstrated good ocular compatibility and may provide sustained drug release for retina.

Keywords: intravitreal drug delivery, sirolimus, porous silicon, rabbit eye, surface chemistry, drug loading and release, sustained release, hydrosilylation, oxidation, silanization

Age-related macular degeneration (AMD) and diabetic retinopathy are the leading causes of blindness in the world and there are very limited treatment options. Wet AMD and proliferative retinopathy are associated with neovascularization because the stressed RPE cells secrete VEGF at the disease site.1,2 Two anti-VEGF agents have been recently approved for these conditions3,4 and require monthly or bimonthly intravitreal injections to neutralize VEGF. VEGF injection frequencies of 6 to 12 times a year remain a major burden on patients and health care providers. In addition, there are two major components of pathologies for wet AMD: excessive VEGF and unwanted cell proliferation and scarring; the current anti-VEGF agents cannot provide the solution to vision-destroying scarring.

In RPE cells, VEGF and other cytokines are induced by the mammalian target of rapamycin (mTOR) or the hypoxia-inducible factor-1 pathway by various growth factors and inflammatory cytokines.5,6 Rapamycin (RAP), also known as sirolimus, prevents mitogen-induced hypoxia-inducible factor-1 and hypoxia-inducible factor-1-dependent transcription and secretion of VEGF5,7 Therefore, RAP may be of use in therapy of wet AMD and proliferative retinopathy. Indeed, preclinical studies have shown that RAP has a therapeutic effect on AMD-like retinopathy.8,9 Due to low solubility of RAP in water (2.6 μg/mL),10 local ocular use is limited. It is also difficult to enhance the solubility of sirolimus by salt generation because of the lack of an ionizable group in the pH range 1 to 10.10,11 Rapamycin is a potent immunosuppressant administered orally for refractory uveitis; however, side effects are associated with systemic use.12

Age-related macular degeneration and diabetic retinopathy are chronic and lifetime diseases. A localized system that maintains an optimal (therapeutic and nontoxic) drug level at the disease site can mitigate the systemic side effects from oral or intravenous administrations.

Porous silicon (pSi) has been reported in recent years as a carrier for the controlled release of drugs.13–15 In contrast to conventional mesoporous silica obtained by sol-gel or precipitation routes, significant control of the pSi microstructure is possible by tuning the electrochemical etching parameters.16,17
In our previous reports, controlled release was achieved by covalent bonding of drugs to pSi particles. However, drug-loading efficiency by covalent bonding was low (<10% by weight). Another useful feature of pSi is its readily modified surface chemistry to optimize the drug loading and release according to the properties of the drug payloads. It has been observed that certain chemistries can slow the degradation of the pSi matrix or enhance the release of poorly soluble active pharmaceutical ingredients.

In the current study, we aimed to develop a novel surface chemistry of pSi for infiltration RAP loading and delivery in the context of ocular use for the treatment of AMD and diabetic retinopathy.

**Materials and Methods**

**Porous Silicon Microparticle Fabrication**

Porous silicon microparticles were prepared by anodic electrochemical etch of highly doped, (100)-oriented, p-type silicon wafers (boron-doped, 1.1 mΩ-cm resistivity; Siltronix, Inc., Archamps, France), in an electrolyte consisting of a 3:1 (vol:vol) solution of 48% aqueous hydrofluoric acid (HF) and ethanol (Fisher-Scientific, Pittsburg, PA, USA) as we previously described. The current density waveform generates a porosity modulation in the pSi layer that displays a sharp peak in the optical reflectance spectrum at approximately 600 nm. The waveform was etched into the silicon wafer for a total of 400 seconds. The resulting porous layer was then removed from the silicon substrate and ultrasonicated in ethanol for 30 minutes in a F55 dual action ultrasonic cleaner (Thermo Fisher Scientific, Pittsburg, PA, USA) to generate the microparticles. Then it was rinsed with ethanol three times and preserved in ethanol.

**Surface Modification of pSi Microparticles**

The freshly made pSi microparticles were modified following three different strategies, as described below.

1. Hydrosilylation of undecylenic acid. The first type of pSi particle, namely pSi-COOH, resulted from chemical modification of freshly etched pSi by microwave-assisted hydrosilylation in the presence of neat undecylenic acid (10-undecenoic acid). This procedure results in a functional pSi surface grafted with C-10 alkyls containing terminal carboxyl groups (COOH). A significant quantity of the surface hydrides (Si-H) remaining after the hydrosilylation reaction were eliminated by air oxidation at 150°C for 48 hours in a ceramic boat inside a muffle furnace (Thermo Fisher Scientific), as previously described. The functionalized samples were kept under vacuum in a desiccator.

2. Hydrosilylation of 1-dodecene. The second type of pSi particle, designated pSi-C12, resulted from chemical modification of freshly etched pSi by microwave-assisted hydrosilylation of 1-dodecene, as previously described. The functionalized samples were kept under vacuum in a desiccator.

3. Silanization of partially oxidized pSi with methoxy(dimethyl)octylsilane. The third type of pSi particle, designated pSiO2-C8, was prepared from pSi by air oxidation followed by covalent grafting of hydrocarbon chains to the porous silica surface with a silanization reaction. Initially, the samples were placed in a ceramic boat inside a muffle furnace at room temperature. The temperature was raised to 600°C at a rate of 10°C per minute, held at 600°C for 1 hour, then slowly cooled to room temperature inside the furnace to minimize the stress in the samples during the transient cooling. This oxidation treatment removes Si-H and partially converts silicon (Si) to silica (SiO2). To generate Si-OH terminated surfaces for further coupling of the organosilane, oxidized pSi particles were treated with 4% (vol/vol) hydrochloric acid aqueous solution (HCl 37% wt%; Sigma-Aldrich Corp., St Louis, MO, USA), shaken for 1 hour at room temperature and washed with deionized water (DI H2O). Approximately 40 mg hydroxyl-terminated pSiO2 microparticles were suspended in ethanol, transferred to a Schlenk flask and dried under vacuum overnight.

**Loading of RAP Into pSi Microparticles**

Drug loading was achieved by the impregnation method using a pressure gradient. Approximately 20 mg functionalized pSi microparticles were transferred to a glass vial, then sealed with a rubber septum and placed under vacuum at less than 0.002 atm for approximately 30 minutes. Thereafter, connection to the vacuum pump was closed and 0.4 mL of an infiltrating solution of 50 g/L RAP (LC Labs, Woburn, MA, USA) in acetone (HPLC grade; Sigma-Aldrich) was injected into the vial. This vacuum-assisted infiltration was used to improve penetration of the drug solution into the pores. Infiltrating liquid flowed rapidly into the pores of pSi microparticles, which were soaked for 10 minutes before breaking the vacuum. The vials were tightly closed with their cap, protected from direct light with aluminum foil wrapping, and placed in a rotator shaker at 20 rpm (position 40, Model 24; Reliable Scientific, Inc., Nesbit, MS, USA) at room temperature. After incubation overnight, the particles were separated from the drug solution by aspirating the supernatant with a pipette, without additional rinsing. The remaining solvent was slowly evaporated under vacuum at room temperature. The dried particles were stored under a protective argon atmosphere until further analysis.

The amount of RAP loaded into pSi microparticles was determined by a solvent extraction method, in which 400 μL acetone (HPLC grade; Sigma-Aldrich) was used to extract RAP from 1 mg pSi particles. The Eppendorf tubes were wrapped with aluminum foil to protect the drug from direct light and were shaken in a rotator at 40 rpm (position 60, Model 24; Reliable Scientific, Inc.) at room temperature overnight. Empty functionalized particles not containing drug were extracted following the same procedure, and were used as a control. For RAP quantitation, both samples and controls were spiked with 1000 ng ascomycin (ASC; LC Laboratories, Woburn, MA, USA) (10 μL of a 100 μg/mL ASC solution in acetone) as an internal standard. Additionally, the empty particle controls were spiked with 1000 ng RAP (LC Laboratories). The pSi microparticles were removed from solution by centrifugation at 10,000 rpm for 10 minutes. The extracts were transferred to an Eppendorf tube, dried, and then suspended in mobile phase A (see below) before injection of 0.001% into a chromatographic column. The internal standard was calibrated by determining, after
Intravitreal Porous Silicon for Delivery of Rapamycin

In Vitro RAP Release and pSi Matrix Degradation

Dynamic drug release from an intravitreal depot was simulated using a syringe pump (NE-1000; New Era Pump Systems, Farmingdale, NY, USA) and a custom chamber with 1.5 mL effective circulation volume to mimic rabbit vitreous. The entire chamber was maintained at 37°C in an orbital shaker (model TSSM1; Chemglass Life Sciences, Vineland, NJ, USA). Samples were sputter-coated with iridium to increase conductivity and improve image quality. Scanning electron microscopy monitored the pSi microparticle surface before functionalization as well as after the drug-loading procedure and further in vitro release using the benchmark crystalline RAP (free drug) as the concurrent control. The drug-release experiment was conducted with a modified dialysis method by using a tubular cellulose dialysis bag (Spectra Por Biotech grade, MWCO 3.5-5 kDa #133192; Spectrum Labs, CA). The mass of pSiO2-C8-RAP microparticles was 5.38 mg (loaded with 1 mg RAP); 1 mg commercial RAP was used as a free drug control. The dialysis bags were immersed in 1.5 mL DI H2O in a glass vial, mimicking the volume of rabbit vitreous. The vials were shaken at 150 rpm and 37°C in an orbital shaker (model TSSM1; Chemglass Life Sciences, Vineland, NJ, USA). The dissolution medium submerging the dialysis bag was sampled and replaced with fresh DI H2O every other day for 19 days. Aliquots were stored at +4°C until further analysis.

Physicochemical Characterization of pSi Microparticles

1. Scanning electron microscopy (SEM). The average particle size and pore size of pSi microparticles were determined from plan-view images of randomly selected particles (n >10) using a Phillips XL30 field emission SEM operating at an accelerating voltage of 5 kV (FEI Phillips, Hillsboro, OR, USA). Samples were sputter-coated with iridium to increase conductivity and improve image quality. Scanning electron microscopy monitored the pSi microparticle surface before functionalization as well as after the drug-loading procedure and the in vitro release studies.

2. Fourier transform infrared (FTIR) spectroscopy. Surface chemistry modifications and drug loading were characterized by FTIR spectroscopy using attenuated total reflectance (ATR) mode on a Nicolet 6700 Smart-TR ATR attachment.

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On analysis of the samples, in addition to quantitate RAP, elemental Si was quantified as Si dissolved in the collected solution by inductively coupled plasma-optical emission spectroscopy (ICP-OES) as reported previously. The purpose is to monitor the relation between drug release and silicon crystal degradation: synchronized drug release and pSi degradation is preferable for intravitreal drug-delivery application.

The optimal formulation of pSiO2-C8 was subjected to further in vitro release using the benchmark crystalline RAP (free drug) as the concurrent control. The drug-release experiment was conducted with a modified dialysis method by using a tubular cellulose dialysis bag (Spectra Por Biotech grade, MWCO 3.5-5 kDa #133192; Spectrum Labs, CA). The mass of pSiO2-C8-RAP microparticles was 5.38 mg (loaded with 1 mg RAP); 1 mg commercial RAP was used as a free drug control. The dialysis bags were immersed in 1.5 mL DI H2O in a glass vial, mimicking the volume of rabbit vitreous. The vials were shaken at 150 rpm and 37°C in an orbital shaker (model TSSM1; Chemglass Life Sciences, Vineland, NJ, USA). The dissolution medium submerging the dialysis bag was sampled and replaced with fresh DI H2O every other day for 19 days. Aliquots were stored at +4°C until further analysis.

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spectrometer (Thermo Fisher Scientific). Sample spectra were collected from 600 to 4000 cm\(^{-1}\) in absorbance mode, with a resolution of 1 cm\(^{-1}\). Each spectrum was the average of 128 scans.

3. Gas sorption analysis. Both surface chemistry modification and drug loading can reduce pSi specific surface area and total pore volume, which was studied using gas sorption analysis. The textural properties of the particles were analyzed by nitrogen adsorption at \(-196^\circ C\) on an ASAP 2020 adsorption analyzer applying a volumetric technique (Micromeritics, Norcross, GA, USA). Approximately 50 mg of the pSi sample was transferred to a preweighed sample tube and degassed at 105°C for a minimum of 12 hours or until the outgas rate was lower than 5 mm Hg per minute. The sample was reweighed to obtain a consistent mass of the degassed pSi. The samples were then manually degassed for at least 2 hours before N\(_2\) isotherm. The specific surface area (m\(^2\)/g) of the particles was calculated from the adsorption branch of the isotherms using the Brunauer-Emmett-Teller (BET) model.\(^{29}\) The pore volume was calculated from a single adsorption point (P/P\(_0\) = 0.99).

4. Elemental analysis. The chemical composition of the samples was analyzed by elemental analysis (CHNS/O system) in a CHNS/O 2400 Series II thermo analyzer (Perkin Elmer, Waltham, MA, USA) to evaluate the mass loading of drug in the pSi carriers.

5. Powder X-ray diffraction (XRD). To assess the status of loaded drug in the pores, powder XRD was carried out using a D8 Advance diffractometer (Bruker, Billerica, MA, USA). Data were collected on powder samples at room temperature using a LynxEye detector (Bruker AXS, Inc., Madison, WI, USA). The X-ray generator was operated at 40 kV and 25 mA using CuK\(\alpha\)\((\lambda = 1.5418 \text{ Å})\), with a scan speed of 0.5 seconds per step, a step size of 0.02°, and a 20 range of 5 to 75°.

In Vitro Cytotoxicity of Released RAP Toward Endothelial EA.hy926 Cells

Cellular assays were performed on human umbilical vein cells (EA.hy926, ATCC CRL-2922; American Type Culture Collection, Manassas, VA, USA) to assess the functional toxicity of released RAP. A WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) toxicity assay was used to test cell viability. The EA.hy926 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM)/F12 (1:1; Gibco Invitrogen, Paisley, UK) and supplemented with 10% fetal bovine serum (Gibco Invitrogen) and 1% antibiotic-antimycotic (100\(\times\); stabilized with 10,000 units penicillin, 10 mg streptomycin, and 25 \(\mu\)g amphotericin B per milliliter [Sigma-Aldrich]) in an incubator (Heraeus Instruments GmbH, Hanau, Germany) for 24 hours to reach 60% to 70% confluence. The cell medium was replaced with 100 \(\mu\)L of a solution of WST-1 (Roche Diagnostics, Indianapolis, IN, USA) was added to each well, and the plates returned to the incubator. The optical density at 440 nm (OD\(_{440}\)) of each well was measured on a UV-Vis spectrophotometer (Spectra Max Plus 384; Molecular Devices, Sunnyvale, CA, USA) at 0.5-hour intervals.
The data were analyzed for the following samples at various concentrations: (a) control RAP, as received, diluted with DI H2O to 1 ng/mL, 10 ng/mL, and 100 ng/mL. A RAP stock solution (1 mg/mL) in dimethylsulfoxide (DMSO, ATCC 4-X, American Type Culture Collection) was used as the starting solution. Final DMSO concentrations were 0.001%, 0.01%, and 0.1%; (b) RAP released from drug-loaded pSi particles, diluted with DI H2O to 1, 10, and 100 ng/mL as the highest concentration. Final RAP concentrations were 0.25 nM, 2.5 nM, and 25 nM, respectively; (c) silicic acid released from non-drug-loaded pSi particles (empty), equivalent in weight to the drug-loaded particles used in (b), following the same dilution strategy as in actual samples.

### General and Ocular Safety in Rabbits

Six pigmented New Zealand Red rabbits (Western Oregon Rabbit Company, Philomath, OR, USA) were used for this study. Only one eye of each rabbit was used for drug injection and the contralateral eye was injected with PBS and served as normal control. All six rabbits had intravitreal injections of 3 mg (2.9 ± 0.39 mg after mass balance) straight etched pSiO2-C8 formulation in 100 µL. Following the intravitreal injections anterior segments of the eyes were monitored by slit-lamp biomicroscopy and the posterior segments of the eyes by indirect ophtalmoscopy. Intraocular pressure and fundus photos of both eyes were recorded on day 4, day 14, and day 56 using Tonopen (Medtronic, Jacksonville, FL, USA) and fundus camera (Canon FA; Canon, Inc., Tokyo, Japan). Three rabbits were killed 2 weeks after the injection as a short-term safety study and the other three were killed 8 weeks as a long-term safety study. Before rabbits were killed, ERG was performed on every rabbit eye. After they were killed, the eye globes were fixed in 4% gluteraldehyde for histology processing. The weight of each animal was recorded immediately before the injection and before they were killed to monitor possible weight loss from the toxicity of the delivery system and the drug.

### Data Analysis

The data from in vitro release using the microdialysis bag were compared between SiO2-C8-RAP and Free RAP using sampling time points as matched pairs with the Wilcoxon signed rank test. Results from the cell viability tests are expressed as means ± SD from at least three independent experiments. The data were analyzed by one-way ANOVA. Multiple comparisons were performed for all pairs using Tukey-Kramer HSD, alpha = 0.05. For IOP and ERG parameters, a paired t-test was used to compare the injected eyes with their untouched contralateral eyes. These tests were performed using JMP statistical software (version 11; SAS Institute, Inc., Cary, NC, USA). The criterion for significance was P less than 0.05 for all the comparisons.

### RESULTS

#### Characterization of pSi Microparticles

The particle dimensions were 43 ± 8 µm in width, 70 ± 16 µm in length, and 25 ± 2 µm thick, as measured by SEM. The pore size was found to be 13 ± 5 nm (Fig. 1).

#### Surface Chemistry and Drug Loading

Hydride terminated freshly etched pSi microparticles were reacted in (1) 1-dodecene to attach hydrophobic, covalently bonded alkyl groups via thermal hydrosilylation (namely Si-C12) (Fig. 2A); (2) reacted in undecylenic acid to attach covalently bonded alkyl groups ending in a carboxyl (-COOH) group via thermal hydrosilylation, followed by thermal oxidation in air at 150°C (namely Si-COOH) (Fig. 2B); and (3) partially oxidized in air at 600°C and modified with hydrophobic covalently bonded alkyl groups via silanization reaction (namely SiO2-C8) (Fig. 2C).

Rapamycin was loaded into the pSi microparticles following surface modification. Drug-loading capacity was quantified by LC-MS and expressed as micrograms RAP per milligrams of silicon. The calculated loading capacity was 36 ± 6 µg/mg (n = 4) for pSi-C12-RAP, 68 ± 8 µg/mg (n = 7) for pSi-COOH-RAP, and 105 ± 18 µg/mg (n = 7) for pSiO2-C8-RAP microparticle formulations. The drug loading was confirmed by measuring the percentage of nitrogen using elemental analysis (CHNS/O). It is assumed that the entire detected content of nitrogen in the sample comes from loaded RAP. In addition, the detected N content for the pure pSi and the functionalized pSi was less than 0.01 wt %. The calculated drug-loading capacity by this method was 17 wt% for pSiC12-RAP, 22 wt% for pSi-COOH-RAP, and 28 wt% for pSiO2-C8-RAP. These values confirm the
successful drug loading and follow a similar trend as compared to those calculated after LC-MS.

Fourier transform infrared spectroscopy also confirmed the surface chemistry treatments of the pSi microparticles as well as the drug loading.

The FTIR spectra of freshly etched pSi as well as functionalized particles are shown in Figure 3A. Freshly etched pSi displays bands that are characteristic of surface hydride species. The band at 2100 cm$^{-1}$ is attributed to the stretching modes of Si-H bonds and the absorbance observed at 910 cm$^{-1}$ is attributed to Si-H deformation modes. After surface functionalization, characteristic bands corresponding to C-H vibrational modes are present in all samples. The bands at 2960 cm$^{-1}$, 2929 cm$^{-1}$, and 2857 cm$^{-1}$ are assigned to stretching modes of C-H bonds, whereas the bands at 1450 cm$^{-1}$ and 1378 cm$^{-1}$ are attributed to deformation modes of C-H bonds. The characteristic stretching mode of a carbonyl group C=O at 1720 cm$^{-1}$ confirms successful hydrosilylation of undecylenic acid. The band at 3412 cm$^{-1}$ is attributed to O-H stretching modes. The hydrogen-terminated surface of the as-prepared pSi particles was converted to silicon dioxide (SiO$_2$) during the thermal oxidation process, as evidenced by the characteristic Si-O-Si absorbance bands at approximately 1069 cm$^{-1}$. After hydrosilylation, the Si-H stretching bands were present, indicating the microparticles were still hydride-coated. Remaining surface hydrides Si-H after hydrosilylation of undecylenic acid were eliminated by air oxidation at 150°C, as confirmed by FTIR. Complete conversion of the Si matrix to SiO$_2$ does not occur under these conditions.

The FTIR spectra of drug-loaded pSi microparticles, namely pSi-C$_{12}$RAP, pSi-COOH-RAP, and pSiO$_2$-C$_8$RAP, as well as the spectrum of pure RAP are shown in Figure 3B. The successful loading of RAP is confirmed by the comparison of the infrared spectra of the functionalized pSi microparticles (Fig. 3A) with the infrared spectrum of RAP-loaded pSi microparticles and pure RAP (Fig. 3B). The intensity of the C-H stretching absorption bands, the C-H deformation bands, and the carbonyl C=O stretching bands are larger in the spectra corresponding to drug-loaded pSi microparticles.

The textural properties of the functionalized and drug-loaded samples were studied by nitrogen adsorption/desorption porosimetry. The isotherms correspond to the functionalized microparticles before and after drug loading are plotted in Figure 4. The isotherms are type IV with H1 hysteresis loops with parallel adsorption and desorption branches, suggesting cylindrical mesopores of approximately constant cross-section. The surface area (S$_{BET}$) and pore volume (V$_p$) of the pSi microparticle formulations as well as the relative polarity of the surface as monitored by the C$_{BET}$ parameter are given in Table 1.

The measured surface area and the total pore volume both decrease after chemical functionalization, and these values are further reduced after drug loading. The observed decreases after functionalization are attributed to the presence of organic groups covalently attached to the matrix surface. The additional decrease in surface area and pore volume after drug loading are consistent with the filling of the inner pore channels with drug.

The C$_{BET}$ parameter is a constant obtained from the BET analysis and is related to the affinity of the solid surface for the adsorbate: the greater the C value, the stronger the interaction. It can be related to the polarity of the samples and it ranges from 80 for the hydrophilic fully oxidized SiO$_2$ sample to 31 for the hydrophobic freshly etched pSi sample.

Powder XRD spectra are shown in Figure 5. Functionalized pSi microparticles show XRD maxima centered at 20 = 28°, 48°, 56°, and 69°, corresponding to reflections from the (111), (220), (311), and (400) crystallographic planes of the cubic diamond lattice of Si. During thermal treatment at 600°C, the pSi skeleton is partially oxidized to SiO$_2$, resulting in a diffuse peak at 20 equals approximately 22° corresponding to amorphous silica.

The main peaks in the powder XRD pattern of crystalline RAP (Fig. 5) appear at 20 = 7.2°, 9.9°, 10.2°, 11.1°, 12.5°, 14.5°, 15.3°, 15.5°, 16.2°, 20.0°, 20.4°, and 21.8°. However, no characteristic RAP peaks were observed from the RAP-loaded pSi samples (Fig. 5), even though the quantity of drug analyzed in each experiment was approximately the same. The XRD peaks observed from the pSi samples all could be assigned to either crystalline Si or to amorphous silica. The results are consistent with the formation of an amorphous

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**Figure 6.** Rapamycin concentration detected by LC-MS/MS from flow chamber in vitro release test in HBSS (black circles, left axis), and cumulative silicon erosion detected as dissolved oxides of silicon by ICP-OES (red squares, right axis), for microparticle formulations pSi-COOH-RAP (left), pSi-C$_{12}$RAP (middle), and pSiO$_2$-C$_8$RAP (right).

**Figure 7.** Concentration of RAP transported across a dialysis membrane as a function of time for crystalline RAP (“Free RAP”) compared with the pSiO$_2$-C$_8$RAP formulation (P = 0.0039, Wilcoxon signed rank test). Solvent was DI H$_2$O and the experiment was maintained at 37°C.
polymorph of RAP within the nanoscale confines of the pSi carriers.

**In Vitro RAP Release and pSi Degradation**

Plotted in Figure 6 are RAP release profiles resulting from in vitro dissolution of pSi-COOH-RAP, pSi-C12-RAP, and pSiO2-C8-RAP microparticle formulations that were performed in a flow chamber with HBSS eluent. Drug release and silicon degradation were monitored simultaneously (Fig. 6).

The drug-release profiles in Figure 6 show that the pSi-COOH-RAP formulation degrades fastest and releases RAP more rapidly than the other two formulations. During the 30-day test period, we measured 60% of the loaded drug had been released and 20% of the pSi matrix dissolved from pSi-COOH-RAP. Drug release from pSiO2-C8-RAP was less extensive, resulting in 14% of the drug being released and 17% pSi

<table>
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<th>Sample</th>
<th>$R^2$</th>
<th>Half-life</th>
<th>$\lambda_z$, d</th>
<th>$T_{\text{max}}$, d</th>
<th>$C_{\text{max}}$, $\mu$g/mL</th>
<th>$C_{\text{last}}$, $\mu$g/mL</th>
<th>AUC$_{\text{last}}$, $\mu$g d/mL</th>
<th>AUC$_{\text{INF, pred}}$, $\mu$g d/mL</th>
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<td>Free-RAP</td>
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<td>73.95</td>
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AUC$_{\text{INF, pred}}$, area under the concentration-time curve from time 0 to the predicted end time point by extrapolation; AUC$_{\text{last}}$, area under the concentration-time curve from time 0 to the last time point; $C_{\text{last}}$, drug concentration at the last observation time point; $C_{\text{max}}$, maximum concentration detected; $T_{\text{max}}$, time at which the concentration was maximum.

**TABLE 2. Pharmacokinetic Parameters for pSiO2-C8-RAP Microparticle Formulation**

**FIGURE 8.** Electrospray ionization in positive ion mode MS spectra (full scan) (A, C) and the ESI-MS/MS spectra of m/z 936.5 [M+Na]$^+$ peak, where M is RAP (B, D) from the release extracts of crystalline RAP (free drug) collected at day 9 from in vitro release following dialysis method. Liquid chromatography MS/MS detection at m/z 936.5 on extracts was performed at 12.38 minutes (A, B) and 12.57 minutes (C, D), corresponding with the retention time of the peak and the shoulder usually observed in the chromatograms. In both cases, ion with m/z 936.5 was observed within these peaks (A, C) with similar MS/MS fingerprints (B, D), confirming that these species are likely isomers of RAP.
Effect of Released Rapamycin on EA.hy926 Cells

Extracts released from the pSiO2-C8-RAP microparticle formulation from the microdialysis experiment were tested on EA.hy926 endothelial cells. The extracts showed significant inhibitory effect in the proliferation assay, relative to control extracts derived from the dissolution of crystalline commercial RAP (Fig. 9; Table 3). These data, along with the LC-MS data, confirmed that the released RAP was in its intact, biologically active form.

Rapamycin derived from the pSiO2-C8-RAP microparticle formulation at a concentration of 2.5 ng/mL or 25 ng/mL showed inhibitory activity equivalent to the medium-dose and high-dose positive controls. The 2.5 ng/mL and 25 ng/mL control samples, which were obtained using the same dialysis setup except using commercial crystalline RAP instead of the microparticle formulation, also showed inhibitory activity equivalent to the medium-dose and high-dose positive controls. The DMSO additive showed no inhibitory effect on cells at concentrations as large as 0.1 % by volume.

In Vivo General and Ocular Safety

Following the intravitreal injections of pSiO2-C8-RAP, no general toxicity or ocular toxicity was observed. The mean body weight for the short-term study animals increased from 2.93 ± 0.15 to 3.23 ± 0.17 kg and from 4.07 ± 0.15 to 4.4 ± 0.25 kg for the long-term study rabbits. The retina was normal and the pSiO2-C8-RAP particles were suspended in clear vitreous humor with clear view of retina and optic nerve (Fig. 10, top). Both IOP and ERGs of the study eyes were comparable with their contralateral eyes (Table 4).

Paraffin section and hematoxylin and eosin staining revealed normal retinal structure for both 2-week animals (Fig. 10, bottom left) and 8-week animals (Fig. 10, bottom right), as compared with their fellow eyes.

Discussion

Rapamycin possesses broad biological activity and is being extensively tested in many clinical trials for various diseases worldwide. Due to its low water solubility and short vitreous half-life, it has potential for ocular use for posterior eye diseases is limited. Most recently, a proprietary RAP depot-forming formulation was reported to be safe for subconjunctival or intravitreal injections and appeared to be effective for uveitis or macular edema. This formulation was reported to provide up to 60 days of sustained release with one administration. Intravitreal depot-forming formulation is likely similar to intravitreal triamcinolone acetonide (TA), which provides a sustained drug release; however, vitreous free drug level is excessively high during most of the release course and can cause adverse ocular events, such as high IOP and cataract formation. Even with 4 mg TA intravitreal injection, the vitreous free drug level reaches above 1000 ng/mL. Similarly, the recent report from the depot-forming sirolimus demonstrated that even a 220-µg intravitreal injection yielded a free drug concentration near 1000 ng/mL. Rapamycin is a very potent drug and high drug concentration could cause adverse effects in a sustained release system. Indeed, a most recent phase I/II clinical trial demonstrated retinal toxicity from intravitreal injection of this proprietary depot-forming sirolimus in eyes with dry AMD.

In the current study, we were aiming at developing a longer and better sustained delivery system for intravitreal sirolimus. We prepared three types of functionalized pSi microparticles, with varying polarity (hydrophobicity/hydrophilicity), as well as hydrolytic stability against dissolution in aqueous media. The
TABLE 3. Connecting Letters Report for Inhibition Assay on EA.hy926 Cells

<table>
<thead>
<tr>
<th>Level</th>
<th>Mean</th>
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</thead>
<tbody>
<tr>
<td>medium_cells</td>
<td>1.6149833</td>
</tr>
<tr>
<td>NoRapSiO2C8_2.5</td>
<td>1.4153250</td>
</tr>
<tr>
<td>medium_DIH2O</td>
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<tr>
<td>nctrl_1</td>
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<td>NoRapSiO2C8_25</td>
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<tr>
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<tr>
<td>nctrl_h</td>
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</tr>
<tr>
<td>Free_2.5ng/mL</td>
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<td>pctrl_h</td>
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<tr>
<td>medium_nocells</td>
<td>0.1434833</td>
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</table>

Levels not connected by same letter are significantly different. Comparisons for all pairs using Tukey-KRAMER HSD, alpha = 0.05. Samples as defined in Figure 9.

**FIGURE 10.** The top two fundus images were from the same rabbit eye that received RAP-loaded pSiO2-C8 particles. Left image was taken 1 week postinjection and the right image taken 8 weeks postinjection, showing clear view of fundus and RAP-loaded pSiO2-C8 particles in vitreous. The numbers of the particles were fewer in the 8-week image but still visible. The histology images from the 2-week (2wks) study (bottom left) and 8-week study (bottom right) were taken at visual streak, which is indicated by high density of ganglion cells. Compared with the left eye (OS), the study eyes (OD) did not show abnormality in retina and choroid. The separation of RPE from underneath choroid in the frame of 8wks OS was caused during tissue processing.
pSi surface functionalization rationale followed in this study involved modifying pSi to make it more hydrophobic to enhance interaction between the hydrophobic RAP drug and the nanocarrier surface for increased drug-loading efficiency. The C_{BET} parameter can be used to rank the order of hydrophilicity: pSiO₂ > pSi-COOH > pSiO₂-C₈ > pSi-C₁₂ (Table 1). Grafting of 1-dodecene (pSi-C₁₂) created a very hydrophobic surface and in comparison, grafting of undecyllic acid (pSi-COOH) created a mildly hydrophobic surface due to the carboxylic acid group and the further partial oxidation process. Finally, silanization of partially oxidized silicon (pSiO₂-C₈) created a slightly more hydrophobic surface than that of pSi-COOH. The oxidized silicon surface of both of these sample types is expected to be hydrophilic, but the grafted alkyl chains impart some hydrophobic character that matches hydrophobicity of RAP.

The surface chemistry had an effect on the efficiency of RAP loading, which followed the following order (μg/mg): pSiO₂-C₈ (105 ± 18) > pSi-COOH (68 ± 8) > pSi-C₁₂ (36 ± 6), as measured by LC-MS. Compared with the covalent drug loading reported previously, the current adsorption RAP loading to pSiO₂-C₈ was also greater and above 10% by weight. The greater capacity of pSiO₂-C₈ for the RAP can be ascribed to the hydrophobic surface chemistry combined with the limited steric hindrance offered by the shorter (C₈) alkyl chains at the surface. Notably, the XRD data show that the loaded RAP is amorphous in all the pSi formulations studied. This suggests that the nanoscale confines of the pSi carriers restrict the RAP precipitate to an amorphous polymorph. This is an important result, because the crystalline nature of many drugs often results in unfavorable or erratic pharmacokinetics behavior. In particular, the ability of the pSi nanostructure to generate an amorphous polymorph of RAP suggests that it can be used to control the free drug availability of a hydrophobic drug. 11,21,40 In the present work, the in vitro dynamic drug release revealed that the availability of the drug for target tissue intake increased by nearly 6-fold (compared with crystalline RAP) when RAP was loaded into the pSiO₂-C₈ particles.

In our previous work, grafting of alkyl groups has been used to covalently attach drug molecules directly to a pSi matrix, and in that case the drug releases at the same rate as the matrix erodes. 22 In the present case, the RAP drug was loaded by infiltration, relying on hydrophobic van der Waals interactions to retain the drug in the porous matrix. The drug-release mechanism from these pSi carriers is thus a combination of diffusion and degradation. Whereas the RAP release profiles are affected by surface chemistry (Fig. 6), they are not as strongly correlated to degradation of the porous matrix as in the systems where the drug is directly bonded to the pSi matrix. We did not observe a burst release of drug in the current systems, probably due to the hydrophobic character of the reservoirs, together with the hydrophobicity of RAP, which does not favor rapid entry of the payload into the aqueous media. The rate of drug release to approximate pSi matrix degradation is an important criterion for the infiltration loaded pSi system; otherwise, empty particles will still be lingering in the vitreous after the therapeutic effect of the payload has disappeared. The data for the pSi-C₁₂-RAP formulation shows that no additional drug is detected after approximately 2 weeks of exposure to the eluent, whereas the dissolved silicon assay indicates that this formulation continuously degrades (by 5%) through the entire 4-week test period. The analysis of the mass spectral data indicated that RAP released from the pSi-C₁₂-RAP formulation also was chemically altered. Thus, it is likely that this formulation also is responsible for the degradation of RAP. It should be pointed out that RAP is itself hydrolytically unstable in aqueous solutions; therefore, all of the measured drug-release values in this work may be underestimated of the actual. The mass spectral data used to determine drug release in Figure 6 quantify only the intact drug molecule and not the degradation products. Of the three formulations studied, pSiO₂-C₈-RAP showed the best performance in terms of drug-loading capacity, minimizing chemical degradation of RAP, and simultaneously releasing active drug with simultaneous carrier degradation. Whereas drug release in the pSi-COOH-RAP system seemed to be dominated by drug leaching, release of RAP from the pSiO₂-C₈-RAP formulation was more tightly correlated with degradation of the pSi matrix (Fig. 6; up to 14% drug released, 17% Si dissolved). We further tested the pSiO₂-C₈-RAP formulation in a refined in vitro cellular inhibition study using a modified dialysis method. Rapamycin is unstable in PBS and HEPES buffer; therefore experiments were carried out in DI H₂O. The released drug showed a positive inhibitory effect on endothelial cells, confirming that this formulation releases the drug in a biologically active form. Consistent with the amorphous nature of the drug within the pSi carrier as discussed above, we found that the availability of the free drug was increased by 6-fold compared with crystalline RAP which has a very low water solubility.

In summary, the current dynamic release study revealed approximately 20% of the total payload leached out within 30 days, which may translate into a sustained release of 150 days. Indeed, the safety study in vivo rabbit eye demonstrated considerable numbers of RAP-loaded pSiO₂-C₈ particles were still present in rabbit vitreous at 8 weeks after a single intravitreal injection of 2.9 (±0.39) mg of the delivery system (containing 305 μg RAP). In addition, with this system a burst release was not observed, which is a precious favorable feature in the drug-delivery arena. This system may provide sustained-long term release of RAP and warrants a full in vivo pharmacodynamic investigation.

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References

| Table 4. Drug-Injected Eyes Versus Their Fellow Eyes for IOP and ERG |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Drug Eyes         | Fellow Eyes       | Difference        | P      | Value |
| ERG b-wave amplitude | 54.14 μV | 53.7 μV | 0.44 | 0.91 |
| ERG a-wave amplitude | -6.62 μV | -5.99 μV | 0.63 | 0.49 |
| IOP | 9.74 mm Hg | 9.89 mm Hg | 0.15 | 0.87 |
Intravitreal Porous Silicon for Delivery of Rapamycin


