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NANOSTRUCTURAL CONTROL OF IMPLANTABLE XEROGELS FOR THE CONTROLLED RELEASE OF BIOMOLECULES

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Introduction

Controlled release systems are designed to deliver controlled amounts of therapeutic agents to specific target sites over extended duration of time. The local delivery eliminates the risks of side effects associated with oral or parenteral therapies such as systemic toxicity. It also improves the efficacy of the treatment by achieving higher drug concentrations at the target site than are possible with systemic administration. In order to avoid the need for a second operation, resorbable and biocompatible materials are very desirable for topical applications during surgery. In response to the need for such carriers, biodegradable polymers have been proposed [1-6]. However, it has been reported that the degradation of polymers, which is the mechanism controlling the release of bioactive molecules, can cause an inflammatory response that interferes with the intended therapy [6-9]. Demineralized bone matrix, synthetic bioactive ceramics and glass-ceramics have also been considered as implantable controlled release materials.[10-16] These materials are biocompatible and can enhance bone healing.

However, these macroporous materials usually demonstrate a poorly controlled "burst" release profile; that is, the release is largely terminated shortly after implantation. For effective healing, however, it is desirable to release an initial large concentration of drugs post-operatively, followed by a steady long-term release.

Sol-gel processed materials represent an alternative to the materials previously used for the controlled release of biologically active agents [17]. Room-temperature processed sol-gel derived silicas (also called xerogels) have been explored for various biomedical applications. Base-catalyzed or two-step acid-base-catalyzed xerogels have been used for the encapsulation of enzymes, cells and living tissues [18-20]. Acid-catalyzed xerogels have been studied as controlled release materials [21-28]. A simple room-temperature process can be used for the incorporation of various biological molecules into silica xerogels. This room-temperature process provides easily reproducible xerogel properties. Large quantities of biological agents can be added and uniformly distributed in the liquid sol. After gelation, condensation and drying, the agents become encapsulated in a glassy solid. These materials are resorbable, highly porous and nanostructured (pore size from 1 to 5 nm). Their structural properties can be extensively controlled by altering sol-gel processing parameters (such as selection of silica precursors, the use of additional oxides, catalysts, solvents, pH of sol, water/alkoxide molar ratio, temperature and conditions of condensation and drying) [17].

In our laboratory, we have studied room temperature processed silica xerogels for controlled release of various biological agents such as drugs, proteins and growth factors. [22-25] In vitro, we studied the effect of synthesis parameters on the structure and the release properties of xerogels. In vivo, we assessed silica xerogels as resorbable

and biocompatible materials [26]. In this paper, we summarize these in vitro and in vivo studies of silica xerogel controlled released materials.

Synthesis of silica xerogels with incorporation of biologically active molecules – methods

The chemical reactions that take place during the synthesis of silica gels include hydrolysis and condensation reactions. The hydrolysis reaction, which can be either acid or base catalyzed, replaces alkoxide groups with hydroxyl groups. Siloxane bonds (Si-O-Si) are formed during subsequent condensation. Alcohol and water are byproducts of the condensation reaction and evaporate during drying. Theoretically, the overall reaction is as follows [17]:

$$n\ \mathrm{Si(OR)4} + 2n\ \mathrm{H2\ O} \rightarrow n\ \mathrm{SiO2} + 4n\ \mathrm{ROH}$$

However, in reality, the completion of the reaction and the chemical composition of the resulting product depend on the excess of water above the stoichiometric H_2O/Si ratio of 2. A number of other sol-gel processing parameters (such as pH of the sol, type and concentration of solvents, temperature, aging and drying schedules, etc.) can also affect the composition, structure, and properties of the resulting product.

In our studies, various biological molecules having different size were used for incorporation into silica xerogels. These included drugs such as naltrexone (0.4 kDa) and the antibiotic vancomycin (3 kDa) and macromolecules such as trypsin inhibitor (20 kDa) and TGF-β (22 kDa) [22-25].

All xerogels were synthesized by a room temperature process using silica precursors such as tetramethoxysilane (TMOS) or tetraethoxysilane (TEOS). In general,

the procedure was as follows: single-step acid-catalyzed hydrolysis (pH 2.0–3.0) with excess of water over the stoichiometric H_2O/Si ratio was used. TMOS (or TEOS), deionized (DI) water and acid were mixed in a glass beaker and stirred using a magnetic stirrer to obtain a homogenous sol. HCl or glacial acetic acids were used as catalysts for the hydrolysis of TMOS and TEOS, respectively. Then molecules of interest were then dissolved in DI and added to the sol. Upon mixing, the sol was cast into cylindrical polystyrene vials. The vials were sealed and the sol samples were allowed to gel and age. Subsequently, the vials were opened, and the gels were allowed to dry until the gel weight became constant.

Using the basic single-step acid catalyzed process, a number of processing parameters was varied to determine the effects on the nanostructure and release properties of the xerogels. These included variations in pH, solvents, water/alkoxide molar ratio, and aging and drying schedule. Modifications of the sol-gel process included the use of organic or organomodified alkoxysilane. Parametrical changes also included variations in concentrations of incorporated bioactive molecules.

Characterization of xerogel nanostructure

Fourier Transform Infrared (FTIR) spectrometry (5 DXC, Nicolet, Madison, WI) and gas (nitrogen) sorption analysis (Autosorb 1, Quantachrome) were used for the xerogel characterization. FTIR analysis was performed using the diffuse reflectance mode. Prior to obtaining nitrogen adsorption-desorption isotherms the samples were outgassed at 35^0C for 48 hours. Multi-point BET [29] was used to determine the surface area (SA), pore volume (PV) and mean pore radius (PR).

FTIR spectra of xerogels (not shown) were typical for acid-catalyzed silica xerogels [17]. The FTIR analysis showed that the xerogels were well hydrolyzed and polymerized [24]. The incorporation of bioactive molecules did not produce any discernible effect on the FTIR spectra of xerogels [24].

Nitrogen adsorption-desorption isotherms of all acid-catalyzed silica xerogels studied were typical for a Type I isotherm (BDDT classification [30]) [24]. This isotherm type is characteristic of a microporous solid, i.e. a solid with a large number of pores having radii equal to or below 1.5 nm. Variation of the processing parameters (which included the use of methanol as a solvent, pH (from 1 to 5), water/alkoxide ratio (from 4 to 10), aging time (from 1 to 14 days), and drying schedule)) did not affect the isotherm type. The isotherms of xerogels containing bioactive molecules such as naltrexone, vancomycin and trypsin inhibitor were also characteristic of a microporous solid.

Physical properties of xerogels such as pore volume, BET surface area, and mean pore radius were derived from the isotherm analysis. Variations in nanostructures of xerogels with water/TMOS ratio and biomolecule load are shown in Tables 1 and 2, respectively.

Table 1. The effect of water/TMOS molar ratio on the surface area (SA), pore volume (PV) and pore radius (PR) of silica xerogels.

Ratio	SA, m^2/g	PV, cc/g	PR, nm
4	519	0.21	0.9
6	761	0.31	0.92
10	822	0.35	0.93

Table 2. The effect of incorporation of vancomycin (V) and trypsin inhibitor (TI) on the physical properties of silica xerogels

Load/sample	SA, m2/g	PV, cc/g	PR, nm
1 mg V	868	0.39	0.98
10 mg V	936	0.48	1.04
2 mg TI	845	n/d	0.96

- n/d: not determined
- vancomycin load/ sample of 1 and 10 mg correspond to 1.1 and 11 mg/g concentration;
- trypsin inhibitor load of 2 mg corresponds to 6 mg/g concentration.

As shown in Table 1, variation of the water/TMOS ratio does not affect the pore size: PR of xerogels with the ratio of 4, 6 or 10 was about 0.9 nm. However, this variation largely affects the surface area (SA) and pore volume (PV): there is a major reduction in the SA and in the PV values with a decrease of the ratio from 10 to 4.

Concerning the incorporation of bioactive molecules, the presence of vancomycin or trypsin inhibitor did not produce a significant effect on the physical properties of xerogels (Table 2). The effect of other parametrical variations (such as the use of methanol as a solvent, changes in pH from 1 to 5, and variations in the aging and drying schedule) was not significant either.

Therefore, it was found that the nanoporosity of xerogels could be varied to a large degree by altering the synthesis parameters such as the water/alkoxide molar ratio.

Controlled release of drugs and proteins from silica xerogels

Xerogels with various concentrations of drugs (naltrexone or vancomycin) or proteins (trypsin inhibitor), were used in the vitro release study. Xerogels with naltrexone were TEOS-derived, while those with vancomycin or trypsin inhibitor were TMOS-derived. Naltrexone was incorporated in xerogel with a water/alkoxide ratio of 6 (R6

xerogel), whereas vancomycin and the trypsin inhibitor were incorporated in R10 xerogels. Xerogel samples used for the study were shaped either as discs or granules. The elution studies were conducted in tris buffered simulated physiological solution (SPS, pH 7.3 at 37^0C) and solutions were exchanged daily. The ratio of each specimen weight to solution volume was large enough to reach solution saturation with Si-species shortly after immersion and thereby prevent degradation of the samples by dissolution [31]. The immersed samples were maintained in a water-jacketed incubator at 37^0C.

The released concentrations of naltrexone and vancomycin were measured using a UV-visible spectrophotometer (Ultrospec Plus, Pharmacia LKB) at 283 and 280 nm, respectively. A gold colloidal assay (Integrated Separation Systems) was used for the determination of concentrations of trypsin inhibitor. Wet chemical analysis (atomic absorption spectrophotometry, Perkin-Elmer 5100 PC, Norwalk, CT) was used to confirm that solution saturation with Si-concentrations was reached within minutes after immersion of xerogel samples .

Controlled release of drugs (naltrexone and vancomycin)

As shown in Figure 1, a sustained release of naltrexone was observed over 12 days. In this figure, the mean cumulative release of naltrexone from R6 xerogels is plotted as a function of elution time and load (3, 6 and 9 mg/sample), The data also demonstrate that the release rate and the amount released were load-dependent. However, the total recovered percentage did not depend on the load and was about 80% for the various load groups.

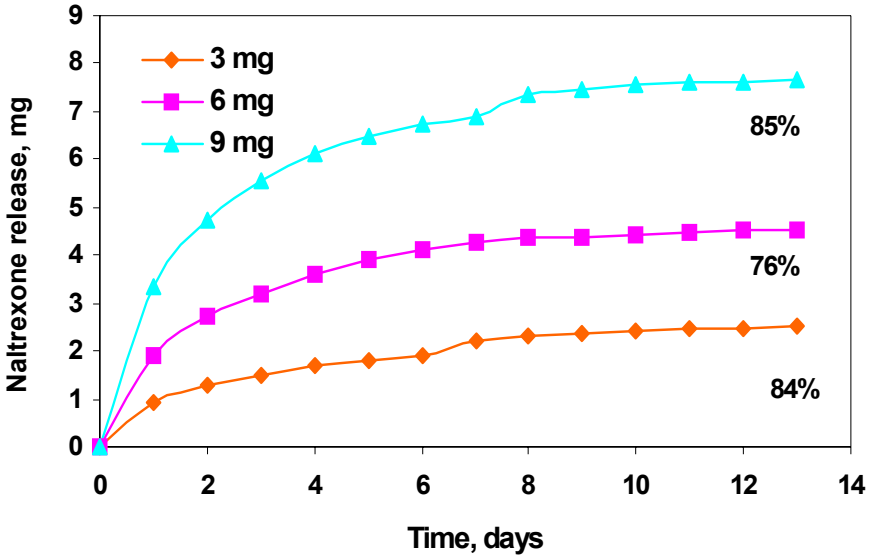

Figure 1. Cumulative release of naltrexone from TEOS-derived xerogel as a function of elution time and load.

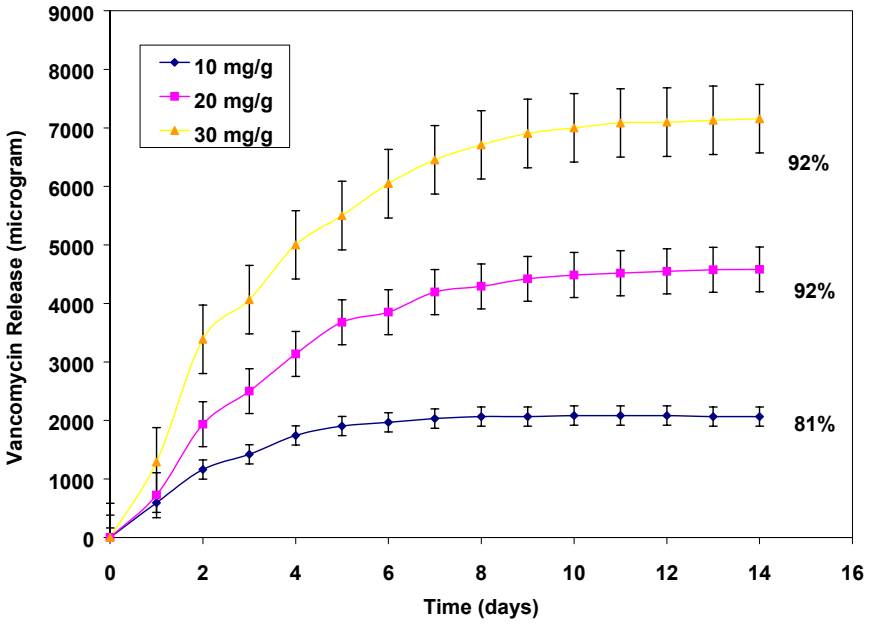

Figure 2. Cumulative release of vancomycin from TMOS-derived xerogel as a function of elution time and load.

The data in Figure 2 demonstrate that the release of vancomycin from R6 xerogels, loaded with 10, 20 or 30 mg/g, was also a controlled, load-dependent, long-term release. The release rates (determined at the linear portions of the release profiles) were 12.4, 21.6, and 34.2 μg/h for the 10-, 20-, and 30-mg/g groups, respectively. These values of hourly release greatly exceeded the minimum inhibitory concentration (MIC) of vancomycin for Gram-positive bacteria (4 μg/ml [32]). By 14 days of immersion, the total release from the 10-mg/g xerogel was about 80% of the original vancomycin load, whereas about 90% was released from 20- and 30-mg/g xerogels.

The possible mechanism of release can be ascertained by presenting the release data as a function of the square root of time [21,23]. Cumulative release of vancomycin from R10 xerogel is presented this way in Figure 3.

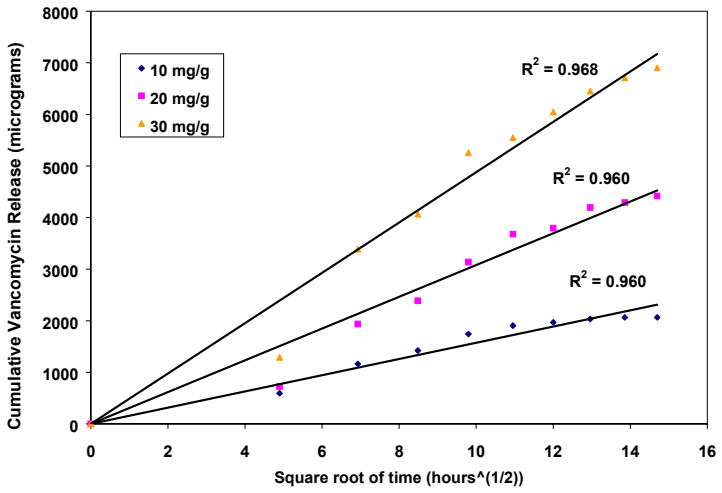

Figure 3. Cumulative release of vancomycin from TMOS-derived R10 xerogel as a function of the square root of time and load.

The linearity of the release plots versus the square root of time reveals first-order release kinetics. As determined by regression analysis, there was no delay and no "burst" in the vancomycin release. Similar presentation of the cumulative release of naltrexone also revealed first-order release without any delay or "burst".

It is well-known that the release which is proportional to the square root of time results from a diffusion driven process. The Highuchi equation relates the total amount of drug released via diffusion from a porous solid matrix into a medium acting as a perfect sink [33]:

$$Q = [(D\varepsilon/\tau) \cdot (2A - \varepsilon C_s) \cdot (C_s t)]^{1/2} \quad (1)$$

where Q is the amount of drug released per unit exposed area after time t, D is the diffusivity of the drug in the permeating fluid, τ is the tortuosity factor of the capillary channel network, A is the total amount of drug present in the matrix per unit volume, C_s is the solubility of the drug in the permeating fluid, and ε is the porosity of the matrix.

The first-order release kinetics of both naltrexone and vancomycin (from R6 and R10 xerogels, respectively) suggests a diffusive mechanism. The model also suggests that the release kinetics depend on the total amount of the drug in the porous matrix. The load-dependent release kinetics of naltrexone and vancomycin agree with the model.

Controlled release of model protein (trypsin inhibitor)

The mean cumulative release of trypsin inhibitor (TI) from R10 xerogels loaded with 2, 5, and 10 mg/sample as a function of elution time is given in Figure 4. The data show a sustained release of TI over a long-term elution period, up to 63 days (9 weeks). Similarly to the release of smaller drug molecules, the release rate of TI and the amount

released were load-dependent. However, in contrast to smaller molecules, the TI release was significantly slower. The total release of vancomycin was about 90% by three weeks, whereas only 7, 20 and 15% of TI was released by this time from the 2, 5 and 10 mg load groups, respectively. Also, the pattern of TI release was different from that of the smaller molecules. The first-order release of both naltrexone and vancomycin occurred without any delay, whereas a significant delay in the TI release was revealed by plotting the data against the square root of time [23]. This delay was load-dependent: 24 or 100 hours for the 10 or 2 mg load groups, respectively. This initial delay was followed by first-order release. By 9 weeks, the total recovery was 21, 43 and 32% for the 2, 5 and 10 mg load groups, respectively.

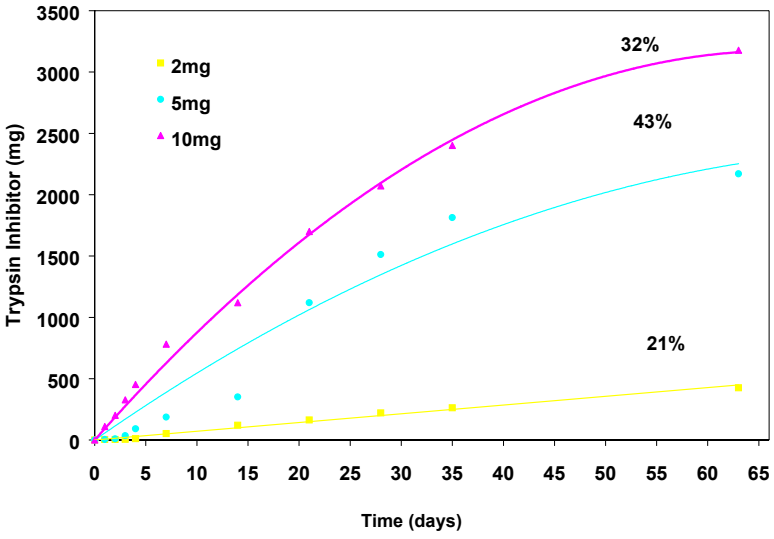

Figure 4. Cumulative release of trypsin inhibitor from TMOS-derived xerogel as a function of elution time and load.

Nanostructural control of release properties

The effect of xerogel nanostructure on the release properties was studied by using xerogels with various water/TMOS molar ratios. As shown in Table 1, variations in the ratio affect the surface area and the pore volume of xerogels without changing their pore size.

Xerogels with the ratio of 4, 6, and 10 (R4, R6, and R10 xerogels) and vancomycin load of 20 mg/g were used. The data in Figure 5 demonstrate a major effect of the ratio on the kinetics of vancomycin release.

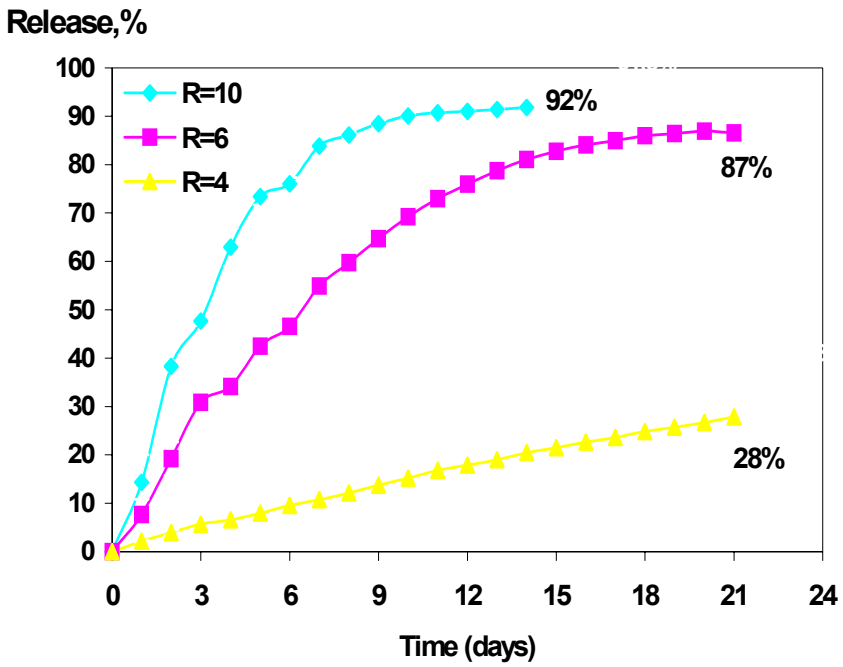

Figure 5. Percent of vancomycin released from TMOS-derived 20-mg/g xerogel as a function of elution time and water/TMOS ratio (R).

The release rates decreased with a decrease in the ratio: the rates of 21.6, 12.2, and 5 μg/h were observed for the R10, R6, and R4 xerogels, respectively (the rates for R10 and R6 xerogels were determined at the linear portions of the plots). About 90% of the vancomycin load was released from R10 and R6 xerogels by 14 and 21 days, respectively. In comparison, only 28% of the load was released from R4 xerogels by 21 days of immersion. Variations in the ratio produced not only a change in the kinetics of release, but also a complete change in the pattern of release. The release from R10 and R6 xerogels was characterized by a first-order release followed by slower, steady release. In comparison, the release from R4 xerogel was a steady, near-zero-order release. This change from a first-order release to a near-zero-order release suggests a change from a diffusion-controlled process to a surface-controlled process.

The observed effect of the water/alkoxide ratio on the release properties is associated with changes in the surface area (SA) and pore volume (PV) (Table 1). In fact, R10 and R6 xerogels with SA above 750 cm^2 and PV greater than 0.32 cc/g, showed a first-order release and a high drug recovery. A major reduction in the SA and PV of R4 xerogels resulted in a major change in the release kinetics of Vancomycin: a slower, zero-order release was observed. These observations suggest a correlation between the porosity of xerogels and the release kinetics. Such a correlation is in agreement with the Higuchi model for the release of a drug from a porous matrix, where the porosity along

with other parameters, such as the solubility, diffusivity, and the amount of the drug in the matrix, affect the release kinetics.

The findings of this study suggest that the long-term release properties of silica xerogels can be tailored via nanostructural control. Desirable structural properties can be selected in view of therapeutic requirements. Control of the structural properties via sol-gel processing is simple and does not produce any adverse effect on the biological activity of the therapeutic agents.

Biological activity of agents released from silica xerogel: Bactericidal efficacy assay of released vancomycin

Vancomycin is the most effective antibiotic against Gram-positive bacteria such as *Staphylococci* and *Streptococci* [32]. The bactericidal efficacy (inhibition of *Staphylococcus aureus* growth*)* of vancomycin released from the xerogel samples, was assayed according to the standard disc-susceptibility procedure [34]. This procedure includes the steps of agar plate inoculation, paper disk impregnation with an antibiotic solution, and testing of the inhibitory effect of the impregnated disc when placed on the inoculated plate. Each elution sample or standard solution was tested in triplicate.

All the elution solutions (collected at 0.5, 1, 3, 7, 14, and 21 days) showed a strong inhibitory effect (Figures 6 and 7). The zones of inhibition are plotted either as a function of release time (Fig. 6) or as a function of vancomycin concentration in solution (Fig. 7). Figure 7 also shows the efficacy of released vancomycin in comparison to that of standard vancomycin solutions. The zones of inhibition significantly exceeded the zone of the MIC of vancomycin for *S. aureus* (4 μg/ml [32]). Focusing on the semi-logarithmic plots in Figure 7, there is a linear relationship between the dimension of the

zone of inhibition and the vancomycin concentration for both solutions tested (the elution solutions and the solutions with calibrated additions of vancomycin). The correlation coefficients, r^2, are equal to 0.995 and 0.999, respectively. This linear relationship is expected in a disc susceptibility test [34]. The overlapping nature of the two sets of data of Figure 7 suggests that the bactericidal efficacy of released vancomycin corresponds to that of the vancomycin solution.

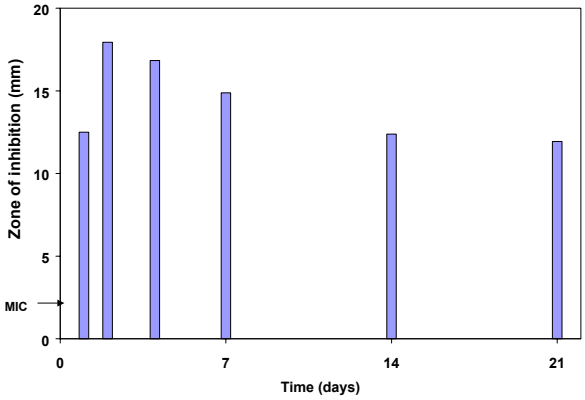

Figure 6. Zone of S. *aureus* inhibition as a function of vancomycin elution time.

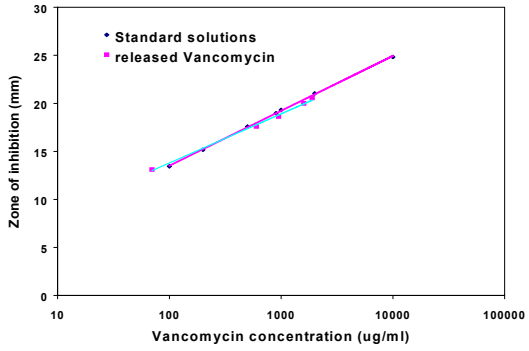

Figure 7. Zone of inhibition of vancomycin released from silica xerogel versus that of standard solutions.

These results provide strong evidence that vancomycin released from silica xerogels fully retains its bactericidal properties. For all molecules studied, we have found that sol-gel processing does not adversely affect the biological activity of molecules incorporated into the xerogels.

In vivo compatibility and resorption behavior of silica xerogels

The tissue response to silica xerogels was determined in the sub-acute implantation phase (up to 4 weeks of implantation) [26]. We correlated the findings to the composition and the resorption rate of the various xerogels. Silica xerogels with and without vancomycin were used .

All xerogels were synthesized by acid-catalyzed hydrolysis of TMOS. Xerogel implants were shaped either as discs, 8 mm in diameter and 2 mm thick, or as granules in the size range of 710-1000 μm. The materials were sterilized by γ-radiation.

Xerogel discs were implanted subcutaneously into the back of New Zealand White rabbits. Additionally, granules were implanted into cylindrical defects (5 mm in diameter and 2 mm in depth) created in the iliac crest. Experimental groups also included controls (sham surgery without implant material). The samples with surrounding tissue were retrieved after either 2 or 4 weeks of implantation. The tissue response was analyzed on thin, stained sections using light microscopy. A total of 34 bone and 38 subcutaneous samples were used for the analysis.

Inflammatory response was scored using a scale from 0 to 4 (0: absent, 1: minimal, 2: mild, 3: moderate, 4: severe) [7]. Morphometric measurements of bone

growth and granule size as a function of implantation time were made using a semiautomatic image analysis system consisting of a high resolution color video camera and Image-Pro Plus analysis software. Statistical analysis was performed using a two-way analysis of variance (ANOVA).

Histological analysis of tissue samples indicated that all subcutaneously implanted discs were encapsulated by a pseudo-synovial membrane of densely packed collagen fibers after 2 weeks of implantation. Xerogel discs showed a minimal inflammatory response, after both 2 and 4 weeks of implantation (the score varied from 0.5 to 1.5).

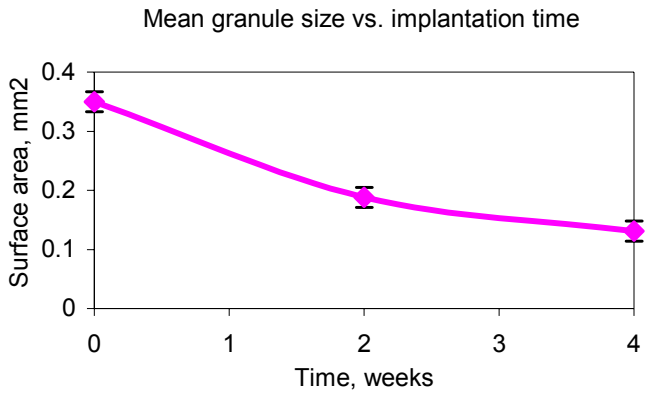

Figure 8. Mean surface area of xerogel granules as a function of implantation time.Error bars represent standard deviation (n=9).

All granules implanted into bone defects showed a gradual decrease in size with implantation time as the morphometric data illustrates in Figure 8. The granule resorption produced a minimal inflammatory response (the score varied from 0.5 to 1.5). The score was lower for granules with vancomycin than for those without it. This resorption was

accompanied by extensive trabecular bone ingrowth. As illustrated by light microscopy microphotographs in Figures 9a and 9b, the bone ingrowth for both the control and the xerogel groups was comparable. The trabecular growth was observed in close vicinity to the granules. The trabeculae were covered with a layer of osteoid tissue and a row of active osteoblasts. The healing of the bone defects via trabecular bone growth, evaluated quantitatively as a ratio of new bone to defect area (Figure 10), was statistically the same for the control and material-filled defects at both 2 and 4 weeks of implantation.

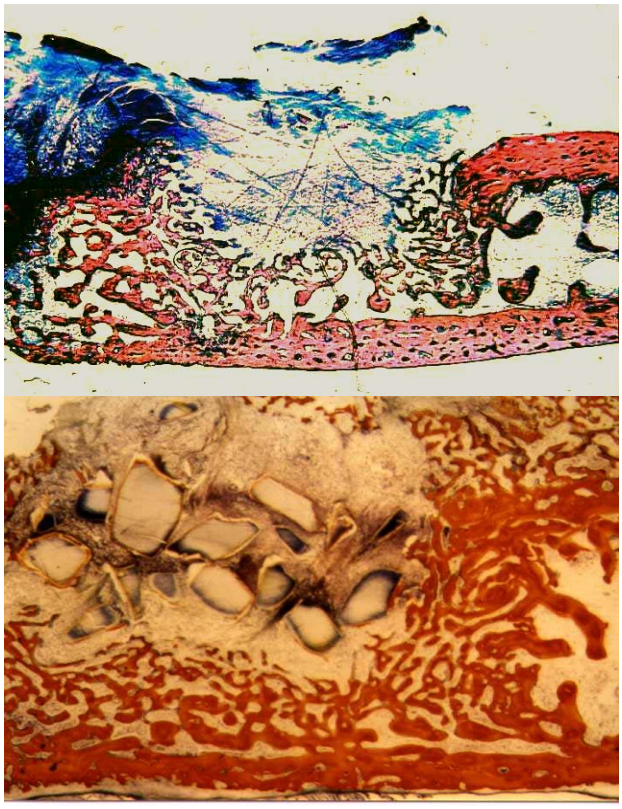

Figure 9 a, b. Micrographs of the bone defects after two weeks of implantation: (a) control and (b) defect with implanted xerogel granules. Extensive trabecular bone ingrowth was observed for both the control and the implant groups.

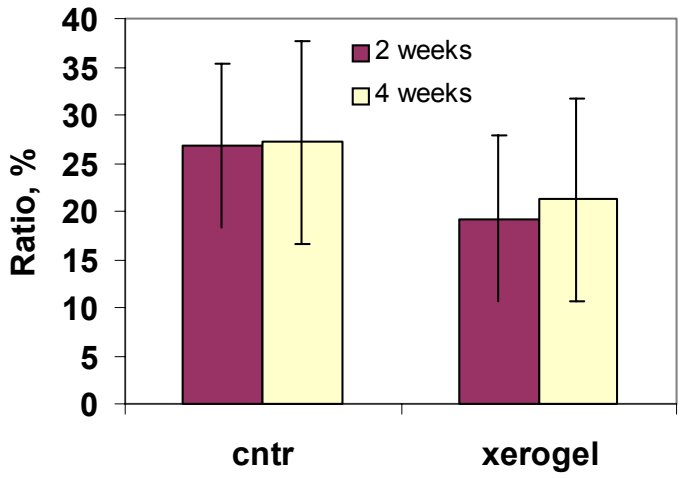

Figure 10. Percent of new bone in the defect as a function of implantation time and experimental groups (control and xerogel). Error bars represent standard deviation (n=9).

This study demonstrates a favorable tissue response to silica xerogels in both subcutaneous and bone sites. Xerogel materials in a granular form showed a gradual resorption, which was accompanied by extensive bone growth. Based on these observations, controlled release silica xerogels are resorbable and biocompatible materials.

Conclusion

Based on the in vitro and the in vivo analyses, silica xerogels can be characterized as resorbable and biocompatible materials for the controlled release of drugs and larger biologically active molecules.

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