Controlled Release of Simvastatin Acid using Cyclodextrin Inclusion System

Masao YOSHINARI¹,², Kenichi MATSUZAKA¹,³, Sadamitsu HASHIMOTO¹,⁴, Kazuyuki ISHIHARA¹,⁵, Takashi INOUE¹,³, Yutaka ODA², Takaharu IDE⁶, and Teruo TANAKA⁶

¹Oral Health Science Center HRC7, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
²Department of Dental Materials Science, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
³Department of Clinical Pathophysiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
⁴Department of Pathology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
⁵Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
⁶Department of Oral Anatomy & Cell Biology, Graduate School of Dental Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Running title: RELEASE OF SIMVASTATIN USING CYCLODEXTRIN

Key words: Simvastatin acid, Cyclodextrin derivative, Controlled drug release

Number of reprints: 100
SYNOPSIS

Simvastatin acid (SVA) has been reported to stimulate bone formation by increasing expression of BMP-2 in osteoblasts. Because of their multi-functional characteristics and bioadaptability, cyclodextrins (CDs) are capable of forming inclusion complexes with many drugs by including a whole drug molecule inside their cavity. In the present study, we prepared SVA/CD inclusion complex solutions with different pHs. These were then used to determine their SVA release properties after coating on titanium substrates and clarify the characteristics of the SVA/CD complexes themselves. The results showed that the lower the pH value of the solution, the lower the release kinetics of the SVA, and that the amount of crystalline complexes in the coatings increased with decrease in pH. These results suggest that the release rate of SVA depends on the pH of the solution and the concomitant crystallinity of the coatings.
INTRODUCTION

Simvastatin (SV), a liposoluble statin [inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase], is widely used for lowering cholesterol levels. Recently, it has been reported to stimulate bone formation, both in vitro and in vivo, in animal osteoporosis models\(^1,2\). This phenomenon was associated with increased expression of the bone morphogenetic protein-2 (BMP-2) gene in osteoblasts. This suggests that controlled release of simvastatin by means of its topical application around dental and maxillofacial implants would promote osteogenesis in surrounding bone tissue\(^3\), and would offer potential in bone regeneration at bone-deficient areas. Furthermore, one distinct advantage of simvastatin for local stimulation of bone formation would be its low manufacturing cost compared to that of direct administration of recombinant proteins such as BMPs. There would also be a reduced possibility of eliciting antibody responses.

In general, SV is administered by gavage, and requires hepatic conversion to metabolically active \(\beta\)-hydroxy acid (SVA) to become medicinally active\(^4,5\). Therefore, for local administration, SV would have to be hydrolyzed to SVA first. We have reported an immobilization method for SVA around implants in order to promote osteogenesis\(^6\). However, a drug delivery system (DDS), in which a carrier was used to deliver SVA to bone-deficient areas, would offer a more powerful strategy for improving bone quality. This would necessitate achieving slow SVA release during the bone formation process after wound healing. Therefore, an effective carrier would be required to express the pharmacological effects of SVA.

Cyclodextrins (CDs), which are recognized as an important group of pharmaceutical excipients, are one such candidate\(^7\). They are cyclic oligosaccharides consisting of (a-1, 4)-linked a-D-glucopyranose units, and have a relatively hydrophobic central cavity and hydrophilic outer surface. The hydrophilic exterior surface of CD molecules makes them
water-soluble, but the hydrophobic cavity provides a microenvironment for appropriately sized nonpolar molecules. They are capable of forming inclusion complexes with many drugs by including a whole drug molecule inside their cavity. In an aqueous solution, the complexes are readily dissociated, and free drug molecules are in relatively rapid dynamic equilibrium with drug molecules bound within the CD cavity8,9,10,11). These noncovalent complexes show new physicochemical characteristics, when compared with the guest molecules, which include better stability, higher aqueous solubility, increased bioavailability, and fewer undesirable side effects. Therefore, if it were possible to form SVA/CD inclusion complexes and control their solubility, it would be possible to control the release properties of SVA.

Since hydrophobic interaction between SVA and CD would be the main mechanism for forming SVA/CD inclusion complexes, it is important to control the hydrophobicity of the guest molecule SVA. SVA hydrophobicity is influenced by pH value, as the characteristic dissociation of carboxyl groups in SVA molecules depends on pH8,12,13). The crystallinity of the inclusion complexes would also influence the release character of the SVA, which would be concomitant to the solubility of SVA/CD complexes.

Consequently, the purpose of the present study was to investigate the possibility of controlling the release rate of SVA. To meet this demand, we prepared SVA/CD solutions with different pH values, and evaluated their SVA release properties from SVA/CD complex coatings on titanium substrates, and clarified the characteristics of the SVA/CD complexes themselves.

**MATERIALS AND METHODS**

*Preparation of SVA*

Commercially available simvastatin \{\((+)-(1\ S,\ 3\ R,\ 7\ S,\ 8\ S,\ 8a\ R)-1,\ 2,\ 3,\ 7,\ 8,\ 8a\text{-}\text{hexahydro}-3,\ 7\text{-}\text{dimethyl}-8\text{-}\text{[2\text{-}\{2R,\ 4R\text{-}tetrahydro-4\text{-}hydroxy-6\text{-}oxo-2H\text{-}pyran-2\text{-}yl\} ethyl\text{-}1-naphthyl}\ 2,\}

---

4
2-dimethylbutanoate, S3449, Wako Pure Chemical Industries, Osaka, Japan} was used in this study. The chemical structures of simvastatin (SV, pro-drug; lactone) and simvastatin acid (SVA, open-acid; β-hydroxy acid) are shown in Fig. 1. Since only the open-ring, or beta-hydroxy acid forms exhibit the efficacy of this medication, SV was hydrolyzed to β-hydroxy acid in the following manner: to open the lactone, 4 mg SV was dissolved in 0.1 mL ethanol (95-100%) and 0.15 mL 0.1M-NaOH was then added. After being heated at 50°C for 2 h, the resulting solution was neutralized with HCl to a pH of approximately 7.2 and brought up to a volume of 1 mL with distilled water.

### Preparation of SVA/CD solution

β-cyclodextrin (CD, Wako Pure Chemical Industries, Osaka, Japan) was used to prepare the inclusion complexes (Fig. 2). 25 mg CD was added to 5mL SVA solution (1000ppm) at 65ºC and stirred; then, the following three solutions, each with a different pH, were prepared by addition of the 0.1N HCl solution. SVA solution (1000 ppm) was also used as a control.

1) SVA/CD 6.8  pH 6.8 (without HCl)
2) SVA/CD 5.2  pH 5.2 (addition of 70 µL 0.1N HCl)
3) SVA/CD 4.2  pH 4.2 (addition of 160 µL 0.1N HCl)
4) SVA (control) pH 7.2

pH 5.2 and pH 4.2 were chosen as occurring just before clouding of the solution at 65ºC, and as slight clouding at 65ºC, respectively.

### SVA release assay from coatings

Surfaces of cp-titanium (Ti) plates (diameter: 30 mm, thickness: 2 mm) were blasted with 50 µm-alumina, and ultrasonically cleaned with acetone and distilled water for 20 min. Surfaces were subjected to ultra-violet radiation before coating to improve wettability. Subsequently, 50
μL each of SVA/CD solution and SVA solution were heated to 65°C and coated onto the titanium surfaces under air-drying at 25°C.

These coated specimens were immersed in distilled water and stored for 0.5, 1, 3, 7 and 21 days at 37°C. At each time interval, absorbance of released SVA in the solution was measured using the UV-Visible spectrophotometer (V-660, JASCO Corporation, Tokyo, Japan) at a wavelength of 239.1 nm\textsuperscript{14,15}. The cumulated concentration of the SVA was calculated using a previously determined working curve.

**Estimation of degree of substitution**

Degree of substitution\textsuperscript{16}, that is, the ability to form inclusion complexes, was estimated to understand the release character of the SVA from the coatings. SVA/CD solutions with different pHs were centrifuged at 3600 rpm at 4°C for 30 min in order to separate the precipitates. After filtering of supernatant fluid with a Millipore filter with a pore size of 0.2 μm, concentration of SVA in the solution was measured using the UV-Visible spectrophotometer, as described above. Degree of substitution was estimated according to the following equation:

Degree of substitution (%) =\text{100 \ (concentration of SVA in original solution - concentration of SVA in supernatant fluid)/ (concentration of SVA in original solution)}

**Characterization of coatings**

The crystallinity of films coated onto the mirror-polished titanium was determined by X-ray diffraction using a thin film attachment (XRD, RINT-2500, Rigaku, Tokyo, Japan), with an X-ray source of Cu-ka1, and at a power of 50 kV/300 mA. These films were coated with the SVA/CD solution, as described in SVA release assay.

The surface morphology of the coatings on the blasted titanium surfaces was observed under a scanning electron microscope (SEM, JSM-6340F, JEOL, Japan). The specimens were
coated with Au-Pd alloy. Accelerating voltage was set at 15.0 kV.

**Statistical analysis**

The data (n=5) were analyzed for statistical significance using an analysis of variance (ANOVA) followed by Scheffe’s test for multiple comparisons.

**RESULTS**

**Release behavior of SVA from coatings on titanium substrates**

Fig.3 shows the SVA release profiles of the films coated onto the titanium substrates using SVA/CD solutions with different pHs. The two-way ANOVA revealed significant differences depending on pH and immersion period (P<0.05). Significant differences in the SVA released were recognized among specimens with SVA > SVA/CD 6.8 > SVA/CD 5.2 > SVA/CD 4.2 in this order at 0.5, 1, 3, 7 days (P<0.05). At 21 days, there was no significant difference in the SVA released between SVA and SVA/CD 6.8 (P>0.05). SVA from SVA-only coatings was almost completely released over one day. Release from SVA/CD-coatings depended on the pH of the solution, with a lower pH not only yielding a lower amount of SVA, but also a tendency toward a slower release. Typically, the concentration of SVA released from the SVA/CD coatings with a pH of 4.2 showed a rate of less than 70% of the entire release on day 21.

**Degree of substitution**

Fig.4 shows UV spectra of the SVA in the original solution and in the supernatant fluid at pH 4.2. No differences in wavelength were observed, with the highest peak occurring at 239.1 nm.

Degree of substitution in each solution is shown in Fig.5. It is estimated that the degree of substitution was 6%, 21%, and 79% for SVA/CD 6.8, SVA/CD 5.2, and SVA/CD 4.2, respectively. A significant difference in the degree of substitution between the SVA/CD 6.8 and
the SVA/CD 5.2 or the SVA/CD 4.2 (p<0.01).

**Characterization of coatings**

X-ray diffraction profiles are shown in Fig.6. The diffraction peaks between 35 and 45 degrees in all specimens were attributed to the titanium substrate. The SVA coatings were almost completely amorphous, whereas the CD coatings showed crystalline patterns at between 3 and 15 degrees with raw β-CD. A clear new crystalline peak, which was not identified by JPCDS cards, was observed at around 28.3 degrees on the SVA/CD coatings. This peak increased with decrease in pH value, whereas the peaks attributed to β-CD decreased with decrease in pH value. This behavior may be explained by the formation of inclusion complexes.

Fig.7 shows SEM images of the titanium substrate (Ti-blasted) and SVA/CD solution coatings with different pHs on the titanium substrates. An irregular morphology was observed in the Ti-blasted specimen. Amorphous-like films covering the Ti-blasted surface were observed in the SVA/CD 6.8 specimen. These films changed to crystalline-like features in the SVA/CD 5.2 and SVA/CD 4.2 specimens. This tendency was marked in the SVA/CD 4.2 specimen.

**DISCUSSION**

The objective of the present study was to clarify the release character of SVA from SVA/CD coatings with different pHs. The results showed that the lower the pH values of the solution, the lower the release kinetics of the SVA, indicating that the release character of SVA can be controlled by adjusting the pH value. Release character is influenced by the degree of substitution and the crystallinity of the coatings.

Degree of substitution plays an important role in balancing CD water solubility and its ability to form complexes. Raising degree of substitution induces binding of guests to CDs by
increasing the surface area available for binding. In this study, the results showed that the lower the pH value of the SVA/CD solution, the higher the degree of substitution. This is a consequence of the different hydrophobic character of the guest molecule, SVA. SVA has a carboxylic acid (pKa = 4.18) which is almost completely dissociated at pH 6.8, with the carboxylic group being gradually deionized with decrease in pH\(^8,16\). Therefore, at a low pH value, SVA becomes stable and hydrophobic, resulting in hydrophobic interaction between SVA and CD being enhanced.

Temperature changes can also affect drug/CD complexes, in terms of degree of substitution. In most cases, increasing the temperature decreases the magnitude of the apparent stability constant of the drug/CD complex, possibly due to subsequent reduction in drug/CD interaction forces such as van der Waals and hydrophobic forces\(^17,18\). In this study, we believe that few SVA/CD complexes occurred at 65°C. However, the number of complexes increased with decrease in temperature during the drying process.

The crystallinity of the SVA/CD complexes influenced the solubility of the coatings\(^8\). A clear crystalline peak was observed by XRD analysis, showing an increase with decrease in pH value. This peak appeared to originate in the inclusion complexes of SVA/CD formed in the coatings. These crystalline structures may have retarded the dissolution rate of the coatings, resulting in delayed release of SVA.

In this study, β-CD was selected as first choice to form inclusion complexes as it has the least solubility in water among the most abundant natural CDs. Further study is necessary to evaluate other CDs that promote slow release of SVA. Hydrophobic CDs, such as alkylated and acylated derivatives, are useful as slow-release carriers in prolonged release formulations of water-soluble drugs\(^19,20,21\).

In conclusion, the results of the present study indicate that the number of SVA/CD complexes formed depended on the pH of the solution, and that subsequent release of SVA
from the coatings depended on the number of complexes and resulting crystallinity of the coatings. These results suggest that SVA/CD complexes offer potential in bone regeneration with a drug delivery system in bone-deficient areas as well as in promoting osteogenesis surrounding bone tissue.

ACKNOWLEDGMENTS

This research was supported partly by the Foundation of Japan Medical Association, by Oral Health Science Center Grant HRC7 from Tokyo Dental College, by a “High-Tech Research Center” Project for Private Universities: matching fund subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006-2011, and by a Grant-in-Aid for Scientific Research (B)(18390524) from the Japan Society for the Promotion of Science. We would like to thank Dr. Setsuo Takeuchi and Yashusi Yoshikawa for their valuable suggestions, and Mr. Jeremy Williams of Tokyo Dental College for his advice on the English of this manuscript.

REFERENCES


Figure captions

Fig.1 Chemical structures of simvastatin (SV) and simvastatin acid (SVA).

Fig.2 Chemical structure (upper) and toroidal shape (lower) of β-cyclodextrin molecule.

Fig.3 Release profiles of SVA from films coated onto titanium substrates using SVA/CD solutions with different pHs (Average + SD). Significant differences in the SVA released were recognized among specimens with SVA > SVA/CD 6.8 > SVA/CD 5.2 > SVA/CD 4.2 in this order at 0.5, 1, 3, 7 days (P<0.05). At 21 days, there was no significant difference in the SVA released between SVA and SVA/CD 6.8 (P>0.05).

Fig.4 UV spectra of SVA in original solution (upper) and SVA in supernatant fluid at pH 4.2 (lower).

Fig.5 Estimated degree of substitution of each solution with different pH (Average ± SD).

Fig.6 Thin film X-ray diffraction profiles of coatings on titanium substrates.

Fig.7 SEM images of titanium substrate (Ti-blasted) and coatings of SVA/CD solutions with different pHs on titanium substrate. Crystalline-like features are recognized in the SVA/CD 5.2 and SVA/CD 4.2 specimens compared to SVA/CD 6.8 specimens (arrows).
Fig. 1

(SV)  

(Hydrolyzed)

(SVA)

Yoshinari
Fig. 2

Yoshinari
Fig. 3

Cumulative released SVA (%)

Immersion time (d)

- SVA
- SVA/CD 6.8
- SVA/CD 5.2
- SVA/CD 4.2
Fig. 5
Fig.6 二段抜き 110mm
Fig.7 二段抜き 140mm

Yoshinari