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Research Article

Release Kinetics and Antibacterial Efficacy of Microporous β-TCP Coatings

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Purpose. The aim of this study was to impregnate microporous β-TCP scaffolds with different antibiotic solutions and to determine their release behavior. *Materials and Methods*. We impregnated a β-TCP scaffold with antibiotics by using three methods: drop, dip, and stream coating with 120 mg/mL of antibiotic solution. After drying for 72 h at 37°C, 2 mL of distilled water was added to the antibiotic-coated plugs and incubated at 37°C. After defined time points (1, 2, 3, 6, 9, and 14 days), the liquid was completely replaced. The extracted liquid was analyzed by capillary zone electrophoresis and the Kirby Bauer disc diffusion test. For statistical analysis, we calculated a mean and standard deviation and carried out an analysis of variance using ANOVA. *Results*. The VAN and CLI release from the β-TCP scaffolds was rapid, occurring within 24 h with 89 ± 0.8% VAN and 90.4 ± 1.5% CLI regardless of the type of insulation. After six days, the VAN and CLI were completely released. All samples taken at later time points had a VAN or CLI concentration below the detection limit of 4 μg/mL. The released amounts of VAN and CLI within the first three days revealed antimicrobial activity.

1. Introduction

Osteoinductive calcium phosphate ceramics are suitable materials for delivery systems [1–5]. This applies in particular to the release of drugs to prevent bone infection. Local drug delivery is especially valuable in association with bone infection since it spares patients the adverse effects of systematically administrated drugs, reduces the risks from resistant bacteria, and enables a high concentration of medication at the infection site [6]. A variety of materials have been used as carriers for the local delivery of antibiotics. These materials are classified as biodegradable or nondegradable. The most commonly used of the non-degradable material is PMMA, which is often impregnated with antibiotics such as gentamycin, clindamycin, or vancomycin. PMMA is not only used as a bone cement or with an antibiotic offset bone cement like PALACOS R+G, but also as antibiotic-impregnated PMMA beads such as SEPTOPAL chains which have been on the market for the last two decades. The major drawback associated with PMMA beads is that they must be surgically removed after exposure to antibiotic release, which usually takes place four weeks after their implantation [7]. The use of biodegradable materials could therefore be advantageous to eliminate the need for a second operation. The most widely used biodegradable materials are polyglycolide and polylactide (PLGA/PLLA). PLGA and PLLA can be used as scaffolds [8], as biodegradable interference screws [9], as coating [5, 10], or as a composite material in combination with other biodegradable materials [11–13]. A disadvantage of polylactide-based materials is that they contain acidic degradation products [14].

Calcium phosphate ceramics (CPC) are suitable in this respect also, since they too are biodegradable [15]. Thus, various attempts have been made to combine CPC, mainly hydroxyapatite (HA) and beta tricalcium phosphate (β-TCP), with antibiotics. Due to its better solubility in water, β-TCP biodegrades faster [16]. HA and β-TCP have often

been used as granules [17] or powder [1, 18], combined with antibiotics. In most cases, the antibiotic was released within 24 to 72 hours [5, 18, 19]. The powders and granules are naturally very mechanically unstable. To produce TCP materials with sufficient mechanical properties, the pores must be small, and the porosity must be reduced to a value approaching 40%. Our group showed that a β-TCP ceramic with 5 μm interconnected micropores and with 40% porosity demonstrates good mechanical stability [20]. It is therefore suitable as a bone substitute material for fixation of cruciate ligaments [20–22] and as a scaffold for bone tissue engineering. Due to the interconnected microporosity, the ceramic is also suitable as a drug release system. Microporous β-TCP ceramics can serve both as a prevention measure and to treat surgical site infections (SSI), because it is mechanically more stable than macroporous β-TCP or granules. We thus loaded microporous ceramic materials with antibiotics for SSI-prevention purposes applying three different methods. We expected the TCP scaffold loaded with antibiotics (AB) to release antibiotics continuously over a period of weeks. We also anticipated that the loading procedure would not trigger a loss of antimicrobial activity. SSI lead to an alteration in the local pH level in the bone [23, 24]. Therefore, all experiments were conducted at a pH value of 5.0 and 7.4 to simulate realistic in vitro conditions

2. Materials and Methods

2.1. Preparation of β-TCP Ceramics. To produce β-TCP plugs, 80 g of α-tricalcium phosphate (α-TCP; $Ca_3(PO_4)_2$) and 20 g of tricalcium phosphate (Art no 102143, Merck, Switzerland; mixture of an apatite and some calcium hydrogen phosphate) were mixed with 60.0 ± 0.2 g of a solution containing 0.2 M Na_2HPO_4 and +1% polyacrylic acid (Art. No 81132, Fluka, Switzerland; Mw = 5.1 kDa). After 2.5 min of intensive stirring, the paste was poured into plastic syringes whose tip had been cut off (Ø = 23 mm). After 45 minutes, the hardened paste was covered with 10 mL of PBS 7.4 solution and incubated for 3 days at 60°C. The samples (Ø = 23 mm; $L \approx 70$ mm) were then dried at the same temperature and sintered at 1250°C for 4 h. Heating and cooling took place at 1°C/min. The cylinders were then machined to obtain plugs of 25 mm long and 10 mm in diameter. The last 2.5 mm of the plugs was given a spherical shape. The samples were then washed in ethanol to remove residual wear particles and calcined at 900°C to burn off all organic residues.

2.2. Characterization of β-TCP Ceramics. X-ray diffraction (XRD) was performed on a Bruker axs D8 Advance X-ray diffractometer (Billerica, USA) using CuK_α radiation at 40 kV and 40 mA. An XRD spectrum was obtained between 20° and 40° (2θ) in 0.01° steps. The XRD spectra of our samples were compared to the standard spectra of β-TCP from the Joint Committee on Powder Diffraction (JCPDS) database (β-TCP = JCPDS 9-169). Porosity was assessed on a POROTEC Pascal 440 (Hofheim, Germany) mercury porosimeter. To detect the morphology and for elementary analysis, we used a PHILIPS XL 30 ESEM FEG (Hamburg, Germany) and an FEI QUANTA 250 FEG (Hillsboro, USA) with EDX unit. All images were taken with a backscattered electron detector. For the ESEM investigations, the β-TCP plugs were broken in the middle with a DUMONT (Montignez, Switzerland) cutting forceps and the breach area was investigated. Because of using ESEM, there was no special coating (carbon or gold) necessary on this breach area to achieve better contrast between ceramics and antibiotics.

2.3. Sample Preparation. Vancomycin- (VAN-) hydrochloride (Cell Pharm, Hannover, Germany) and Clindamycin (CLI) 2-phosphate (MP Biomedicals, Illkirch, France) solution with concentrations of 40, 80, and 120 mg/mL were prepared with deionized water. The β-TCP scaffolds were divided using a diamond band saw Exakt 310 (Exakt, Norderstedt, Germany) into segments of 3 mm ×Ø10 mm. These segments were washed in pure ethanol (Carl Roth, Germany) to remove the sawdust and dried at 37°C for 24 h. Then they were weighed with a Scaltec SBA 32 scale (Goettingen, Germany) and stored in a compartment dryer at 37°C. Ten samples were used for each coating method. The loading experiments were carried out in triplicate.

Dip Coating. Dip coating was done in 24-well cell culture plates with 2 mL quantities of 40, 80, and 120 mg/mL VAN and CLI solutions. The scaffolds were incubated in the solutions for 24 h at room temperature (RT), transferred into other 24-well cell culture plates, and dried for 72 h at 37°C.

Drop Coating. Drop coating was done in 24-well cell culture plates. A drop of 125 μL of the 40, 80, and 120 mg/mL VAN or CLI solutions was placed on the front of the cylindrical scaffold. The 24-well plates were sealed with Parafilm M (Pechiney Plastic Packaging, Chicago, USA). After 2 h of incubation at RT, the scaffold was turned over and a further 125 μL drop of 40, 80 and 120 mg/mL or VAN CLI solution was placed on the other front. After 2 h of incubation at RT, the scaffolds were placed in a clean 24 well plate and dried for 72 h at 37°C.

Stream Coating. A third coating method was used to coat the TCP scaffolds with antibiotics. Because of its setup, we call this method "stream coating." The scaffolds were put in a 5 mL syringe. The punch was pulled out and the ceramic scaffold was placed directly onto the syringe outlet. Then the scaffold was overlaid with 5 mL of antibiotic solution. Four mL of the solution was pressed through the scaffold, so that a constant supernatant of VAN solution remained beyond the scaffold. Pressures were increased by a 0.45 μm syringe filter. The scaffolds were then transferred to clean 24 well plates (TPP) and dried at 37°C for 72 h in a compartment dryer.

All samples were briefly swilled in deionized water after the drying procedure to remove the AB from the outer surface. The antibiotics' load amounts for each coating method were determined by weight in triplicate.

2.4. Drug Release. To assess the amount of antibiotic loaded, the drug-loaded scaffolds were weighed again and placed in 24-well plates. Two unloaded scaffolds were used as blanks.

Each 24-well plate was filled with 2 mL of deionized water, sealed with PARAFILM, and incubated at 37°C. All tests were conducted at pH values of 7.4 and 5.0. To setup a pH of 5.0, 0.1 M HCl was used. After 1, 2, 3, 6, 9, and 14 days, the scaffolds were removed and placed into new 24-well plates filled with 2 mL of deionized water. The solutions thus obtained were stored at −4°C for examination by capillary zone electrophoresis (CZE) and disc diffusion method (DDM).

2.5. Capillary Zone Electrophoresis. CZE experiments were performed on a Beckman Coulter P/ACE MDQ Molecular Characterization System (Brea, USA) with a 40.5 cm fused silica capillary (31 cm to detector) and 50 μm inner diameter. The analytic conditions were: field strength: 20 kV, temperature during measurement: 25°C, and injection pressure: 2 psi for 6 s. The detection wavelengths were 221 nm (VAN) and 200 nm (CLI). We prepared fresh running buffers, citric acid buffer, pH 3.2 to detect VAN and borate buffer, pH 9.2 to detect CLI [25]. The capillary was flushed for 6 min with 0.1 M NaOH, 6 min with deionized water, and 6 min with running buffer at a pressure of 20 psi before injecting each sample. All samples were measured in triplicate by UV.

2.6. Disc Diffusion Method. The released amounts of VAN and CLI were tested with a standard NCCLS disc diffusion test. DDM was performed on Mueller-Hinton agar (Carl Roth, Germany). A suspension of *Staphylococcus aureus* ATCC 25923 with a 0.5 McFarland standard was placed on the Mueller-Hinton agar. OXOID paper discs (Wesel, Germany) were moistened with 10 μL sample liquid and placed on Mueller Hinton agar. OXOID paper discs with 5 and 30 μg VAN and 2 and 10 μg CLI were used as standards. After 24 h incubation at 37°C, the zone diameters were measured in the usual manner.

2.7. Statistical Analysis. Origin 8.5.1 Professional (OriginLab Corporation, Northhampton, USA) was used for graphic presentations and statistical analysis. A probability of error (P) of less than 0.05 was defined as significant. All samples were measured in triplicate. Normal distribution was ascertained by means of the Shapiro-Wilk-Test. The Mann-Whitney Test was used to compare mean values.

3. Results

3.1. Characterization of β-TCP Ceramics. The EDX spectrum (Figure 1) shows that the chemical composition on the surface of the samples is made up of only Ca, P, and O atoms, as would be expected in calcium phosphate ceramics such as β-TCP. A quantification of the EDX spectra (only Ca and P without standards and with theoretical k-factors) results in 60 at% Ca and 40 at% P, that is, a Ca/P ratio of 1.5 and this is equal to TCP. Figure 2 shows an XRD pattern of our β-TCP sample in powder form (b) and the β-TCP standard from the JCPDS database (β-TCP = JCPDS 9-169) (a). The comparison of the two XRD spectra reveals a clear consensus with no signs of a further phase or a shift. Using mercury porosimetry we measured a mean pore radius of 2.44 ± 0.47 micron and a total porosity of 46 ± 1%. Figure 3 illustrates the pore size distribution. We also found a small amount of nanoporosity; the pore size distribution in Figure 3 shows a peak at 100 nm. Figure 4 contains an ESEM image of this nanoporosity.

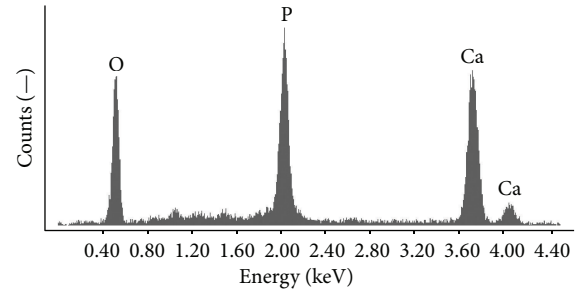


Figure 1: EDX spectrum of β-TCP sample; spectrum taken with Philips ESEM XL 30 FEG with 12 kV accelerator voltage and 100 sec lifetime counting period.

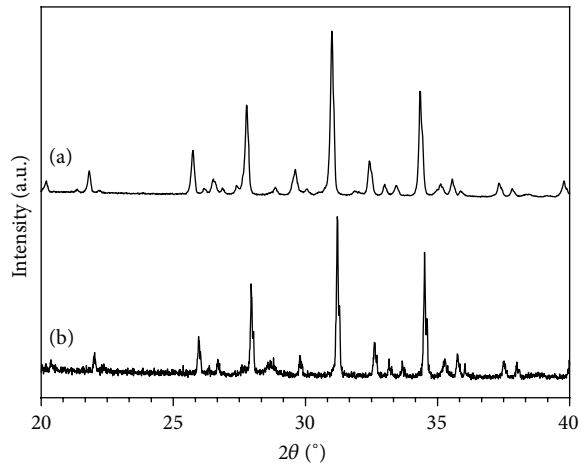


Figure 2: XRD patterns of the standard for β-TCP from JCPDS database (β-TCP = JCPDS 9-169) (a) and microporous β-TCP sample (b).

3.2. Coating Experiments. The average weight of the β-TCP scaffolds before coating was 0.438 g ± 0.059 g (N = 90). Table 1 summarizes our release-experiment results. The drop coating methods produced the maximum charge amount in all cases. The loading method we developed, in terms of load quantity, is in the second place. The dip coating method demonstrated the lowest load rate and took, compared to the other methods, the longest time to complete.

3.3. Coated AB Examination via ESEM/EDX. Studies using ESEM and EDX demonstrated that the antibiotic not only adheres to the outer surface of the test specimens, but also penetrates the core of the microporous ceramic. Figures 5 and 6 shows ESEM images that verify the diffusion of a substance into the porosities of the β-TCP scaffolds. The lighter structures are β-TCP and the darker areas represent VAN or CLI. Because we used a sensor for backscattered electrons, the brightness of the calcium phosphate ceramics in the image

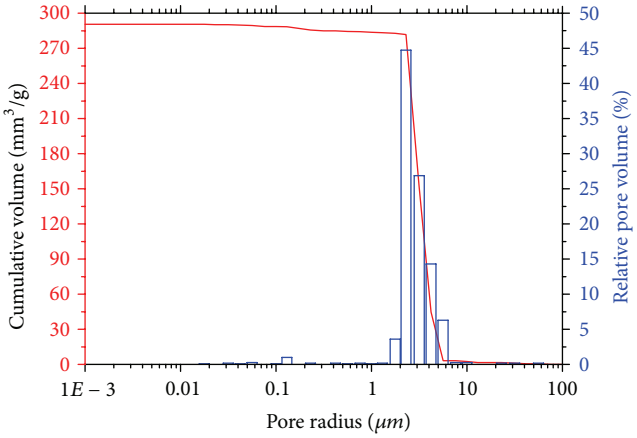


Figure 3: Pore size distribution of microporous β-TCP ceramics.

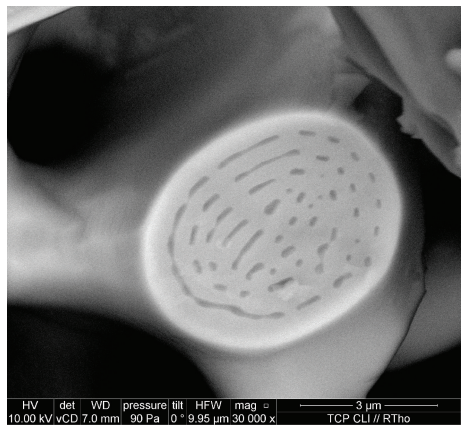


Figure 4: Nanoporosity of β-TCP ceramics. Image taken with a backscattered electron detector, 10 kV accelerator voltage, pressure of 90 Pa, and magnification 30000x.

Table 1: Comparison of coating methods, quantities of antibiotics in the porous ceramics, and load duration ($N = 10$).

Coating	Load quantity of AB [26] achieved with AB solution of			Load duration
	40 mg/mL	80 mg/mL	120 mg/mL	
Drop coating	9.8 ± 2.3	14.7 ± 3.7	29.5 ± 2.5	4 h
Stream coating	6.7 ± 1.3	11 ± 2.7	20.6 ± 3.6	5 min
Dip coating	9.4 ± 0.79	9.4 ± 1.9	16.1 ± 2.7	1 d

is an indication of the higher atomic number of Ca. As the antibiotics mainly consist of carbon, they appear darker in the photograph. The white bars in Figure 6 reveal VAN's penetration depths into the porous ceramics in case of dip and drop coating. The penetration depth of drop-coated ceramics was on average 1000 μm ±127 μm and of dip-coated ceramics a mean 350 μm ±68 μm. The EDX spectra in Figure 7 confirm this by virtue of the presence of carbon, chloride and sulfur, as VAN was available as VAN-HCl; we thus used the chloride as a marker element. Sulphur was employed as the elemental marker for CLI. Carbon is not found in the scaffold material in the quantities identified here, and chloride and sulphur never occur. In the EDX measurements we identified traces of carbon detected in the unloaded β-TCP samples. Those could be carbon residues from the microporous ceramics' manufacturing process. Further ESEM measurements were taken to ascertain how deeply VAN and CLI diffuse into the scaffold to rule out the possibility that the antibiotics were located exclusively on the scaffolds' perimeter.

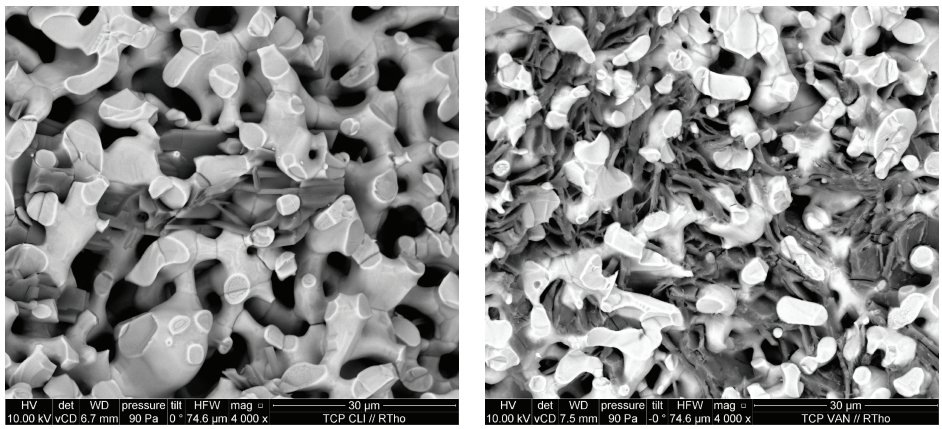
Table 2: Recovery rate of VAN using CZE ($N = 10$).

Coating method	Quantity of AB in scaffold (mg)	Quantity of AB recovered from scaffold by CZE (mg)	Recovery rate (%)
Drop coating pH 7.4 (VAN)	29.5 ± 2.5	27.2	92.2
Drop coating pH 5.0 (VAN)	29.5 ± 2.5	29.1	98.6
Dip coating pH 7.4 (VAN)	16.6 ± 1.3	16.04	96.6
Dip coating pH 5.0 (VAN)	16.1 ± 2.7	14.5	89.8
Stream coating pH 7.4 (VAN)	20.6 ± 3.6	19.5	94.5
Drop coating pH 7.4 (CLI)	22.2 ± 2.8	22.0	99.1
Dip coating pH 7.4 (CLI)	23.5 ± 2.9	23.4	99.6

3.4. Drug Release. These drug release experiments were conducted using 24-well cell culture plates with deionized water at pH 5.0, corresponding to the altered pH in the presence of inflammation, and pH 7.4. However, we detected no significant difference between the two pH values in the quantities of VAN released. The drip-coated scaffolds showed greater release of VAN during the first 24 h because of the higher quantity of VAN available: 29.5 ± 2.5 mg was transferred to the scaffolds and 26.3 ± 2.9 mg was released within 24 h. In contrast, the dip-coated scaffolds were loaded with 16.1 ± 2.7 mg VAN and released 14.3 ± 0.8 mg VAN within 24 h (Figure 8). The stream coating method achieved a load of 20.8 ± 3.6 mg VAN, and 16.9 ± 2.5 mg of VAN was released after 24 h.

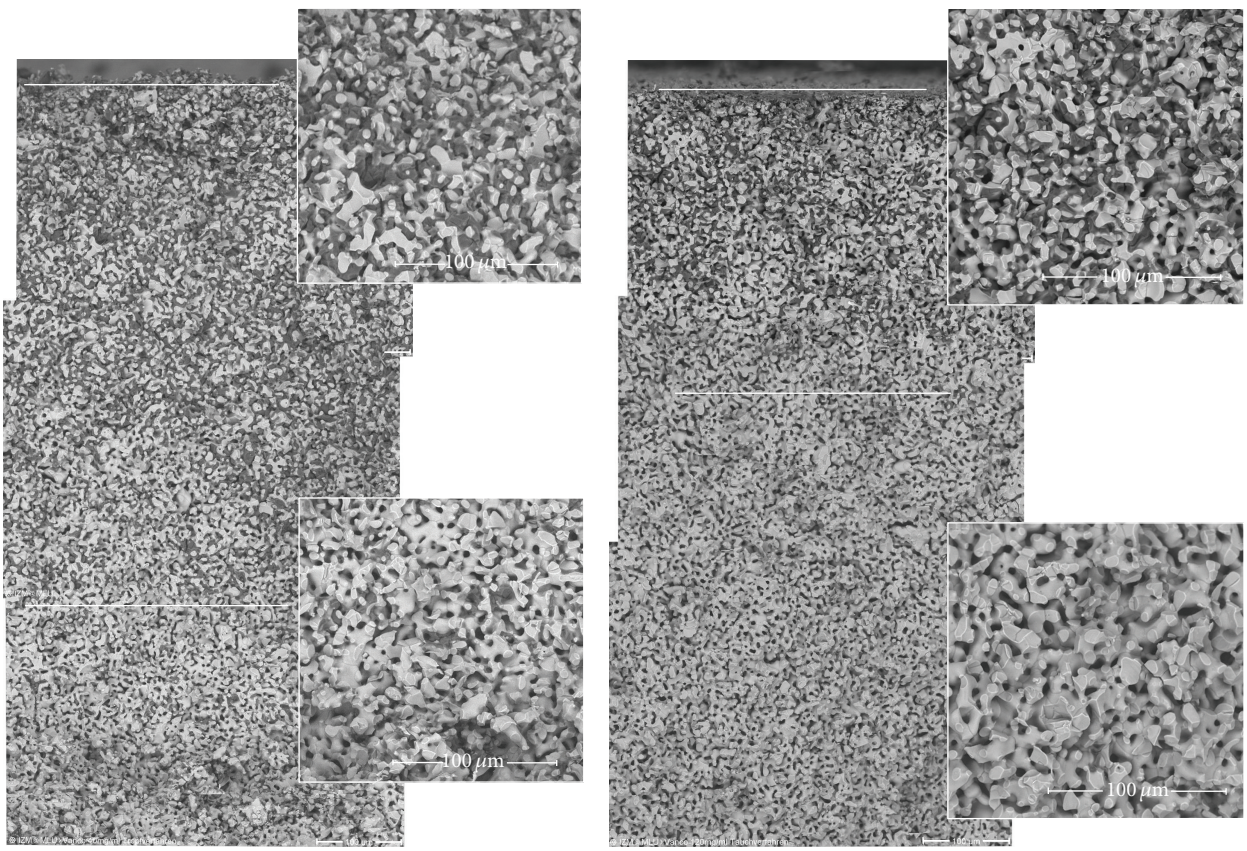
VAN was released rapidly at both pH values: 88.25 ± 3.6% within 24 h. The release of CLI was also rapid: 90.4 ± 1.5% within 24 h. The VAN and CLI releases were complete after 6 days. All samples taken at later time points had a VAN concentration below the detection limit of 1 μg/mL or a CLI concentration below the detection limit of 2 μg/mL. The proportion of released VAN recovered by means of CZE was 94.3 ± 4.1%, and for CLI 99.4 ± 0.4%.

A comparison of the coated and recovered quantities of VAN and CLI is shown in Table 2.



(a) (b)

Figure 5: ESEM images of β-TCP impregnated (dip coating) with antibiotics. (a) CLI coating; (b) VAN coating. Images taken with a backscattered electron detector, 10 kV accelerator voltage, pressure of 90 Pa, and magnification 4000x; the calcium phosphate ceramic appears (due to Ca's higher atomic number) brighter than the antibiotics, which consist mainly of hydrocarbons. All images were taken from the breach area.



(a) (b)

Figure 6: ESEM images of β-TCP. Comparison of VAN's penetration depth into the ceramics: breach area of cylindrical ceramics, breach along boresight, (a) drop coating; (b) dip coating; each image is a combination of three individual images taken with PHILIPS ESEM XL 30 FEG using a backscattered electron detector, 12 kV accelerator voltage, pressure of 100 Pa, magnification 500x, and the calcium phosphate ceramic appears (due to Ca's higher atomic number) brighter than the antibiotics, which consist mainly of hydrocarbons; top: frontal area (where the drop was placed in case of drop coating) bottom: center of ceramic cylinder and the small images are enlargements of image sections; the upper enlargement displays filled porosity and the lower empty porosity.

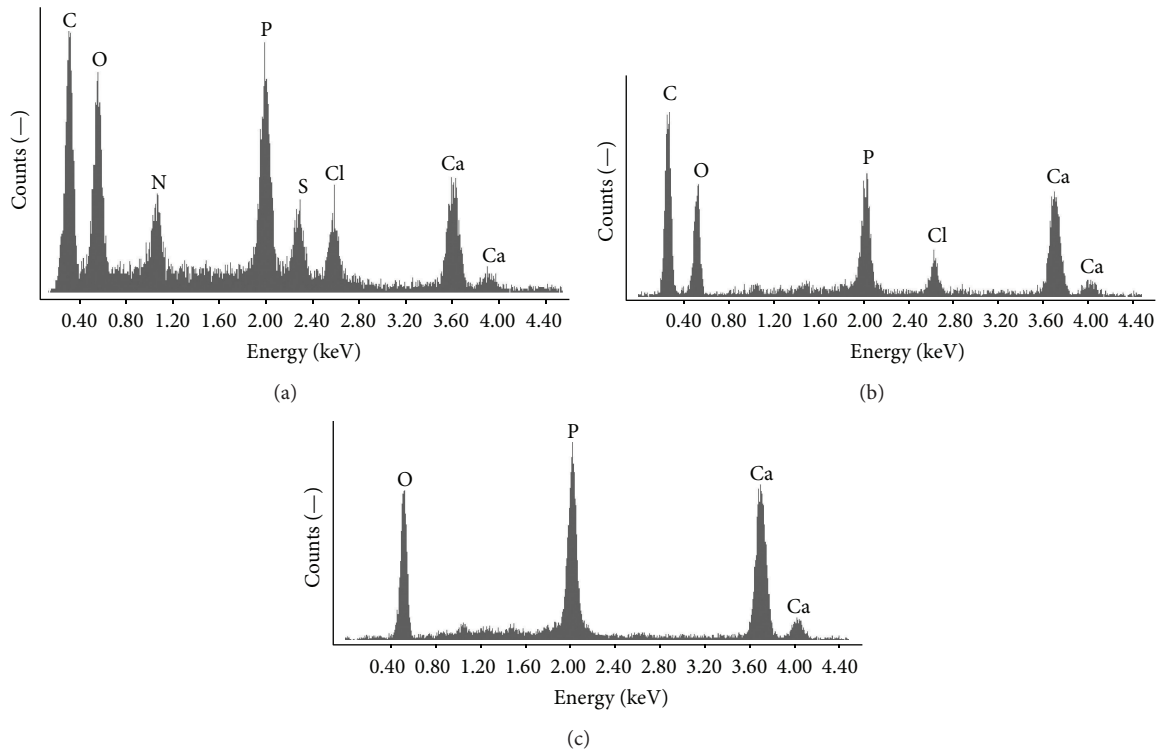


Figure 7: EDX spectra of clindamycin (a) and vancomycin (b) compared to β-TCP (c); spectra taken with a PHILIPS XL 30 FEG ESEM with EDX unit, with 12 kV accelerator voltage and 100 sec lifetime counting period.

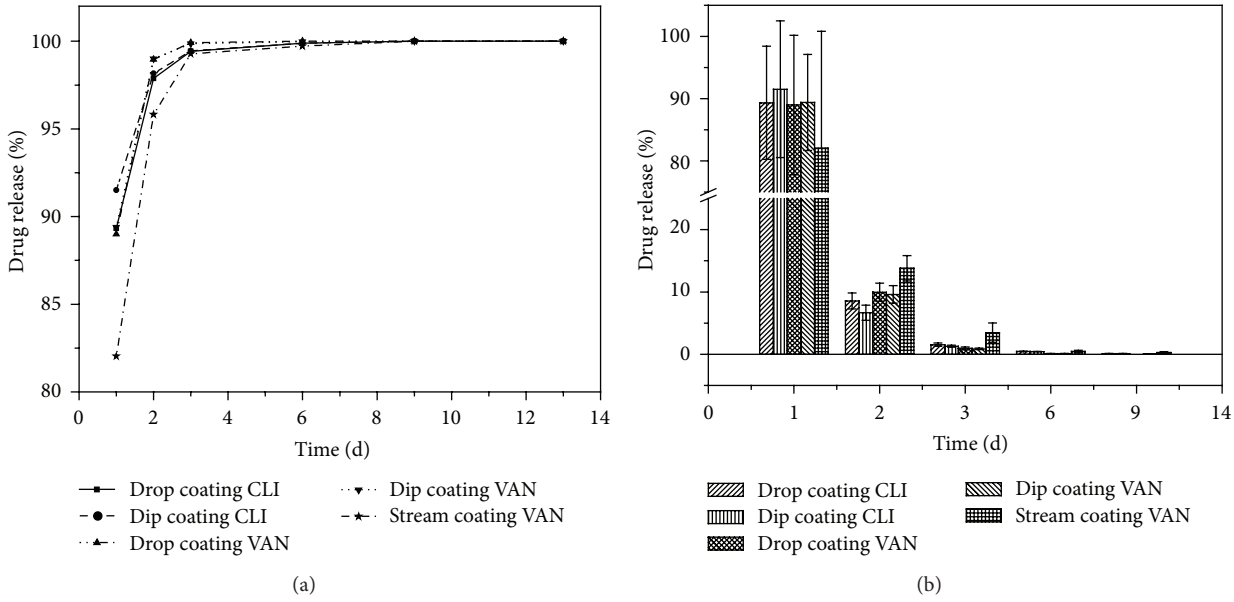


Figure 8: Comparison of release kinetics: (a) cumulative release; (b) daily release.

3.5. Disc Diffusion Method. The antimicrobial activity of the released VAN is shown in Table 3. We observed antimicrobial activity only on the first 3 days; at later times, the amounts released were below the MIC [27, 28] and also below the detection limit of 1 μg/mL [29].

4. Discussion

Elemental analyses proved unequivocally that the samples consist of pure phased beta-TCP. This material has also been used in previous studies [20, 21, 30] because it is more highly

Table 3: Released VAN concentrations and inhibition circles at different time points ($N = 3$).

Day of sampling	Vancomycin concentration (mg/mL) detected via CZE	Inhibition circle diameter (cm)
1	28.37	1.97 ± 0.23
2	2.66	1.8 ± 0.1
3	0.20	0.97 ± 0.06
6	0.02	0 ± 0
9	0.003	0 ± 0
13	0	0 ± 0

water-soluble than hydroxylapatite [16]. The sintered molded body of β-TCP exhibited a microporous structure, while in the literature β-TCP only as granules or as a powder [1, 31], incorporated in calcium phosphate cement [32, 33] or in the form of a macroporous scaffold such as macroporous DePuy Synthes chronOS.

With regard to filling the ceramics using ESEM and EDX, we detected antibiotics within the microporous ceramic; such antibiotic penetration has not been demonstrated before, as so far, the antibiotic has only been demonstrated on the ceramic's surface or barely under it.

If one compares how we loaded the ceramic with the dip coating described by Y. Zhang and M. Zhang [34] and Alkhraisat et al. [35], one observes that stream coating is superior to the latter in terms of the time it takes to load while loading the same amount of antibiotic.

(See Table 1) Hofmann et al. [33] describe results similar to ours, namely a burst of vancomycin release within the first 24 hours. Examining calcium sulfate cements, Hesaraki et al. [36] observed similar release characteristics as we did in our experiments, which barely released anything after 72 h.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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