

Surface-bound bisphosphonates enhance screw fixation in rats—increasing effect up to 8 weeks after insertion

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Submitted 05-11-14. Accepted 06-09-24

Background A bisphosphonate coating improves screw fixation 2 weeks after implantation in cancellous bone. This study on rats examined further development of fixation over time for screws inserted in cancellous and cortical bone.

Methods SS screws were coated with a multiple layer of fibrinogen. Half of the screws were coated further with bisphosphonates, which were linked to the fibrinogen. The screws were inserted in cancellous and cortical bone in rats. The rats were killed after 5 h, 4 days, 1, 2, 4, 8, and 24 weeks, and fixation was evaluated by pull-out test.

Results There was a gradual increase in pull-out force over time in both cancellous and cortical bone. The bisphosphonate coating improved fixation. Moreover, the difference between the bisphosphonate and control groups increased with time. The pull-out force was almost twice that of the controls for screws inserted in cancellous bone at 8 weeks. Energy uptake was increased more than 3-fold.

Discussion The energy uptake and pull-out force of a screw depends on the bone engaged with the threads. Thus, the presence of bisphosphonates increased the amount or quality of this bone by affecting the resorption/formation in a positive way. The increased effect of the bisphosphonates with time thus suggests that bisphosphonate is retained within the remodeling bone, with a positive effect on its gradual adaptation to the implant.

Early fixation of an implant is important for its long-term survival (Mjöberg 1997). Thus, it is important to enable high uptake of mechanical load as early as possible, in order to prevent micro-motion and thereby decrease the thickness of the fibrous capsule. For example, early stability of a total knee replacement is a strong predictor of late loosening (Ryd 1992) and dental implants are mostly load-protected for a few months after insertion to allow osseointegration and later toleration of very high loads. It is therefore of some importance to try to achieve enhanced early mechanical fixation of implants in bone.

We have shown previously that stainless steel screws with a bisphosphonate coating improve the mechanical fixation of implants in rat tibia, as measured at 2 weeks (Tengvall et al. 2004). In principle, bisphosphonates could exert this effect in two different ways: The first way would be by blocking the early bone resorption that is initiated by the tissue damage at surgery (Dhert et al. 1998). This would be most prominent in cortical bone, which is known to resorb in areas with osteocyte damage (Verborgt et al. 2002, Noble et al. 2003) such as in the vicinity of an implant. The other way would be by affecting the new bone that is forming and remodeling around the implant. Bisphosphonates may influence this by changing the balance between formation and resorption, such that a larger or stronger envelope of bone is formed around the implant. This would

imply a stronger and longer-lasting effect than if only early bone loss was inhibited. However, it would require that the bisphosphonate is somehow retained in the area. In order to look for such a long-term effect, we investigated the development of the mechanical fixation of bisphosphonate-coated screws inserted in cancellous and cortical bone up for to 24 weeks.

Material and methods

We studied the mechanical fixation of stainless steel screws in 5 different experiments: (1) the effect of acid etching, (2) cancellous vs. cortical bone, (3) time sequence in cancellous bone, (4) time sequence in cortical bone, and (5) long-term effect. For all studies, the preparation of the bisphosphonate-coated screws was done according to the same protocol. A multilayer of fibrinogen was covalently bound to the screws, followed by application of two types of bisphosphonates. In order to estimate the thickness of the coated film on the screws, flat silicon surfaces were treated according to the same protocol (except for hydrogen fluoride etching). The procedure for the animal operations was also the same, except for the distance from the physis to the insertion site.

Animal experiments

40 rats were used in the study on the effect of acid etching, 20 in the study comparing cancellous and cortical bone, 91 in the study on time sequence in cancellous bone, 106 in the study on time sequence in cortical bone, and 21 in the study on long-term effects. Male Sprague-Dawley (SD) rats were used with a mean body weight of 390 (271–520) g. The animals were kept 2 per cage and given free access to rat chow and water. Institutional guidelines for the care and treatment of laboratory animals were followed and the study was approved by the regional ethics board.

Surgical procedure

Surgical equipment was sterilized in an autoclave. Sterile gloves, theater caps, gowns, and surgical masks were used. The rats were anesthetized with isoflurane gas. Each rat received a subcutaneous injection of 7 mg oxytetracycline and 0.05

mg buprenorphine. One of the legs (or both in the study on cancellous vs. cortical bone) was shaved and cleaned with chlorhexidine alcohol. The rat was placed in a sterile surgical glove and a hole in the glove was cut, through which the shaved leg was pulled out. Sterile tape was wrapped around the paw and the leg cleaned once more with chlorhexidine alcohol.

A 5–6 mm longitudinal incision was made along the medial aspect of the rat tibia. The periosteum was reflected dorsally to the physis. A hole was made in cancellous bone with a hand-held 1.2-mm drill, 3–6 mm from the proximal physis. In cortical bone a 1.3-mm hole was drilled with an air-driven drill, 7–11 mm from the physis. A screw was inserted and the skin sutured. The treatment was randomized by a lottery so that it would be performed blind by the operating surgeon (except when the screw was inserted in cortical or cancellous bone in the study on cancellous vs. cortical bone).

Mechanical analysis

All analyses were performed while blinded. The rats were killed after 5 h, 4 days or 1, 2, 4, 8, and 24 weeks. The screws were tested for pull-out strength in a computerized materials testing machine (100 R; DDL Inc., Eden Prairie, MN), at a speed of 0.2 mm/s (Skripitz and Aspenberg 2001). The tibias were mounted with the head of the screw pointing out through a hole (diameter 3.5 mm) in a metal plate, and the head of the screw was fixed by a metal pin that passed through a hole in the screw head and a horseshoe-shaped connector.

Preparation of stainless-steel screws

Stainless-steel screws, measuring 1.7 mm in diameter (type M 1.7) and 3 mm in length, were used. This type of screw has been used in several previous studies on implant fixation (Skoglund et al. 2004, Tengvall et al. 2004). The heads of the screws were designed with a hole, so that they could be fastened to a hook in a materials testing machine.

The screws were (except for some controls) etched in acid to create a micro-porous surface, and then a layer of cross-linked fibrinogen was bound to the surface to act as a slow-release polymer. Pamidronate was then linked to the fibrinogen

and ibandronate adsorbed to it. In all experiments (except the one on the effect of acid etching), the controls had the fibrinogen coating but not the bisphosphonates.

The screws were etched for 40 min in 40% HF, cleaned twice in a basic hydrogen peroxide solution (5:1:1 proportions of H₂O, 30% H₂O₂, and 25% NH₄OH) at 85°C for 5 min and finally cleaned in an acidic hydrogen peroxide solution (6:1:1 proportions of H₂O, 30% H₂O₂, and 37% H₂SO₄) at room temperature for 1 min. After each cleaning step, the screws were rinsed extensively in distilled water.

Water was exchanged for an organic solution through stepwise rinsing of the screws in ethanol, acetone, and xylene. Silanization was performed in xylene with 1% H₂N(CH₂)₃Si(OC₂H₅)₃ (APTES; from ABCR, Germany) for 2 h. Weakly attached and excess silane was removed by ultrasonication in xylene for 30 s followed by 3 rinses in xylene. The screws were dried in a flow of N₂ and incubated at room temperature for 30 min in freshly prepared 6% glutaraldehyde, HOC(CH₂)₃CHO, in 0.2 M Tris buffer, pH 9. The rest of the procedure is described elsewhere (Tengvall et al. 2004; however, fibrinogen concentration was 10 times higher). Briefly, glutaraldehyde was bound to the screws to serve as an anchor for fibrinogen attachment. 10 layers of fibrinogen were applied by EDS/NHS coupling chemistry, followed by chemical binding of pamidronate (pamidronate dissolved in water, 1 mg/mL Aredia; Novartis, Sweden) by the same technique. Finally, ibandronate was weakly bound to the screws by overnight incubation (ibandronate dissolved in water, 50 µg/mL Bondronate; Roche, Switzerland). This procedure was used to maximize the uptake of bisphosphonate. The screws were dried in a flow of N₂ and placed in plastic tubes filled with N₂, which were then sealed with plastic film and stored until the screws were used for insertion in rats.

The thicknesses of the organic film were estimated with an ellipsometer (Auto-EI-III; Rudolph Research, Hackettstown, NJ) by measurement in air, using flat silicon surfaces as a model for the screw surface (because of the difficulty in performing these experiments on the curved and rough stainless steel). The data are, however, believed to closely reflect those using stainless steel. Measure-

ments were made on 4 surfaces, which were handled according to the same protocol as described above—except for the HF-etching. Thicknesses of the coated layers were calculated according to the McCrackin (1969) evaluation algorithm, and converted into an approximate adsorbed amount per unit area using de Feijter's formula (1978). The assumed refractive index (n_f) of the protein and bisphosphonate was 1.465. The ellipsometric thicknesses of 1 nm of adsorbed molecules correspond to approximately 0.12 µg/cm² (Tengvall et al. 2004). For actual thicknesses, see Results.

Statistics

Comparison between two independent groups was performed with an unpaired t-test. In the time sequence studies, the variances appeared to be proportional to the mean values; thus, the data was ln-transformed before a two-way ANOVA was done, using treatment and time as independent factors. Results from ellipsometry were based on more than 10 repeated measurements per surface, and on 2–4 surfaces. Mean values of molecular film thickness were calculated for each surface, and the mean of the 2–4 surfaces was then calculated.

Results

Complications

6 of 278 rats in total died during, or shortly after anesthesia. 16 rats were excluded due to failures during operation or analysis. 11 of 106 rats in the study on time sequence in cortical bone were excluded due to unclear identification. In 18 rats in the time-sequence study and 1 rat in the long-term effects study, a subcutaneous abscess was observed around the head of the screw. These rats were excluded. We did measure them as well, however, and found no difference in mechanical fixation from the rats that were included. 1 rat in the long-term effects study was killed within 6 weeks postoperatively due to the size of the abscess. All exclusions were performed while blinded.

Surface organic film

The thicknesses of the organic film layers and the corresponding surface mass densities on silicon are given in Table 1.

Table 1. Ellipsometric film thicknesses and surface mass densities of fibrinogen and the two bisphosphonates pamidronate and ibandronate on silicon

	Film thickness, Å		Surface mass density, µg/cm ²
	mean	SD	
Fibrinogen	840	68	10.1
Pamidronate	30	18	0.36
Ibandronate	10	4	0.12

Table 2. Biomechanical data for the study on the effect of acid etching

	Treatment	n	Mean	SD
Force (N)	Blank	12	38	7
	Acid etched	11	38	4
	Bisphosphonates	10	50	15
Stiffness (N/mm)	Blank	12	65	23
	Acid etched	11	79	15
	Bisphosphonates	10	113	21
Energy (Nmm)	Blank	12	23	7
	Acid etched	11	21	5
	Bisphosphonates	10	24	10

Effect of acid etching

Blank, etched or bisphosphonate-coated screws were inserted unilaterally into one of the tibial cortices. The rats were killed and tested at 2 weeks (Table 2). No differences in pull-out force, stiffness, and energy were observed between blank and etched screws (Table 3). However, there was a difference in pull-out force between bisphosphonate-coated screws and both blank screws ($p = 0.01$) and etched screws ($p = 0.01$).

Table 4. Biomechanical data for each group in the study on cancellous vs. cortical bone

Bone structure	Treatment	Biomechanical data			
		n	mean	SD	
<i>Force (N)</i>					
Cancellous	Control	9	40	4.8	
	Bisphosphonates	9	46	6.3	
Cortical	Control	7	56	16	
	Bisphosphonates	7	73	9.1	
<i>Stiffness (N/mm)</i>					
Cancellous	Control	9	84	34	
	Bisphosphonates	9	85	32	
Cortical	Control	7	114	54	
	Bisphosphonates	7	141	48	
<i>Energy (Nmm)</i>					
Cancellous	Control	9	20	6.5	
	Bisphosphonates	9	27	8.8	
Cortical	Control	7	29	7.8	
	Bisphosphonates	7	37	12	

Cancellous bone vs. cortical bone

Bisphosphonate-coated and control screws were inserted bilaterally into cancellous or cortical bone so that each animal had either two cancellous screws or two cortical screws. One of the screws was a control and the other one was bisphosphonate-coated. The screws were pulled out after 2 weeks (Table 4). Bisphosphonate coating had the effect of increasing the pull-out force in both cortical ($p = 0.03$) and cancellous bone ($p = 0.02$). No significant difference in the effect of treatment was observed between cancellous bone and cortical bone (Table 5).

Time sequence in cancellous bone

Pull-out force, stiffness, and energy increased with time in both controls and bisphosphonate-coated screws ($p < 0.001$; Table 6). Bisphosphonate coat-

Table 3. The effects of acid etching and bisphosphonate coating of the screws, expressed as 95% confidence intervals for the difference between mean values in group comparisons. P-values are based on unpaired t-tests

	Acid etched vs. blank			Bisphosphonates vs. blank			Bisphosphonates vs. acid etched		
	mean	(95% CI)	P-value	mean	(95% CI)	P-value	mean	(95% CI)	P-value
Force (N)	0	(-12 to 12)	1.0	32	(5 to 58)	0.01	32	(6.3 to 57)	0.01
Stiffness (N/mm)	22	(-2.7 to 46)	0.1	74	(46 to 102)	< 0.001	43	(23 to 63)	< 0.001
Energy (Nmm)	-8.7	(-33 to 14)	0.5	4.3	(-28 to 36)	0.8	14	(-18 to 47)	0.4

Table 5. The effect of bisphosphonate-coated screws in cancellous vs. cortical bone described as the 95% confidence interval for the difference between means, expressed as percentage increase from controls. The p-values are based on unpaired t-tests

Bone structure		Percentage increase as a result of bisphosphonate treatment, mean (95% CI)		P-value	Treatment × bone structure ^a
Force (N)	Cancellous	15	(2.1 to 28)	0.02	0.2
	Cortical	30	(6.0 to 55)	0.03	
Stiffness (Nmm)	Cancellous	1.2	(–35 to 38)	0.9	0.6
	Cortical	24	(–23 to 71)	0.3	
Energy (Nmm)	Cancellous	35	(–0.8 to 71)	0.09	0.6
	Cortical	28	(–9 to 64)	0.2	

^a P-values for the interrelationship between effect of treatment and bone structure were calculated with unpaired t-test on the bisphosphonate/control ratio in bilateral experiments in a cancellous bone group vs. the same ratio in a cortical bone group.

Table 6. The effect of bisphosphonate and the effect of time in cancellous bone, described with p-values based on 2-way ANOVA (ln-transformed)

	Time	Treatment	Time × treatment ^a
Force (N)	< 0.001	< 0.001	0.02
Stiffness (N/mm)	< 0.001	0.004	0.4
Energy (Nmm)	< 0.001	< 0.001	< 0.001

^a P-values for the interrelationship between treatment and time (i.e. whether effect of treatment changed significantly changed with time).

ing gave increased pull-out force ($p < 0.001$). The effect of the treatment also increased over time (pull-out force: $p = 0.02$). Pull-out force had increased significantly at 4 and 8 weeks, with up to a doubling at 8 weeks (Table 7). Energy and stiffness had increased significantly at 8 weeks, with up to 3 times the energy.

Time sequence in cortical bone

The results were similar to those for cancellous bone, but with higher initial values. Pull-out force, stiffness, and energy increased with time in both the controls and the bisphosphonate-coated screws ($p < 0.001$; Table 8). The pull-out force was higher in bisphosphonate-coated screws ($p < 0.001$). The effect of the treatment also increased over time

Table 7. Biomechanical data for the study on time sequence in cancellous bone

		Biomechanical data			Percentage increase as a result of bisphosphonate mean (95% CI)	
Time	Treatment	n	mean	SD		
<i>Force (N)</i>						
1	B	10	32	13	19	(−13 to 50)
1	C	11	27	4.9		
2	B	8	62	21	27	(−5.7 to 59)
2	C	9	49	9.4		
4	B	6	120	37	79	(31 to 127)
4	C	7	67	17		
8	B	8	170	53	102	(50 to 154)
8	C	10	84	38		
<i>Stiffness (N/mm)</i>						
1	B	10	55	17	3.8	(−19 to 26)
1	C	11	53	9		
2	B	8	90	17	20	(−5.5 to 46)
2	C	9	75	23		
4	B	6	122	19	25	(−2.4 to 51)
4	C	7	98	29		
8	B	8	144	19	30	(12 to 48)
8	C	10	111	24		
<i>Energy (Nmm)</i>						
1	B	10	16	4.8	6.7	(−19 to 32)
1	C	11	15	4.1		
2	B	8	35	14	25	(−13 to 63)
2	C	9	28	6.3		
4	B	6	45	34	32	(−49 to 114)
4	C	7	34	6.6		
8	B	8	113	34	197	(122 to 273)
8	C	9	38	25		

B – Bisphosphonates; C – Control

Table 8. The effect of bisphosphonate and the effect of time in the cortical bone study, described with p-values based on 2-way ANOVA (ln transformed)

	Time	Treatment	Time x treatment ^a
Force (N)	< 0.001	< 0.001	< 0.001
Stiffness (N/mm)	< 0.001	0.03	< 0.001
Energy (Nmm)	< 0.001	< 0.001	< 0.001

^a P-values show the interrelationship between treatment and time (i.e. whether effect of treatment changed significantly with time).

Table 9. Biomechanical data for the study on time sequence in cortical bone

			Biomechanical data			Percentage increase as a result of bisphosphonate	
Time	Treatment	n	mean	SD	mean	(95% CI)	
Force (N)							
0.5	B	10	51	5.6	6.3	(−10 to 23)	
0.5	C	9	48	11			
1	B	10	59	14	−11	(−27 to 6.1)	
1	C	10	66	11			
2	B	11	94	16	29	(9.3 to 48)	
2	C	11	73	18			
4	B	5	132	23	69	(32 to 106)	
4	C	6	78	26			
8	B	9	133	26	43	(16 to 70)	
8	C	7	93	25			
Stiffness (N/mm)							
0.5	B	10	91	15	−5.2	(−17 to 6.5)	
0.5	C	9	96	9.7			
1	B	10	90	21	−4.3	(−20 to 11)	
1	C	10	94	14			
2	B	11	104	19	24	(7.2 to 40)	
2	C	11	84	14			
4	B	5	151	8.2	43	(32 to 53)	
4	C	6	106	11			
8	B	9	140	22	−4.8	(−17 to 7.6)	
8	C	7	147	15			
Energy (Nmm)							
0.5	B	10	22	5.2	10	(−17 to 37)	
0.5	C	9	20	6.5			
1	B	10	29	8.0	−6.5	(−28 to 16)	
1	C	10	31	7.5			
2	B	11	61	13	45	(19 to 71)	
2	C	11	42	13			
4	B	5	97	30	126	(52 to 199)	
4	C	6	43	22			
8	B	9	90	52	114	(26 to 203)	
8	C	7	42	20			

B – Bisphosphonates; C – Control

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Table 10. Biomechanical data for the study on long-term effects

	Treatment	n	Mean	SD
Force (N)	Bisphosphonate	9	229	52
	Control	10	184	40
Stiffness (N/mm)	Bisphosphonate	9	320	36
	Control	10	285	47
Energy (Nmm)	Bisphosphonate	9	109	42
	Control	10	80	24

Table 11. 95% confidence intervals for the difference between mean values in group comparison in the long-term effect study. P-values are based on unpaired t-tests

	Bisphosphonates versus control		
	mean	(95% CI)	P-value
Force (N)	25	(1.6 to 47)	0.05
Stiffness (N/mm)	12	(–0.9 to 25)	0.09
Energy (Nmm)	36	(–3 to 75)	0.08

(pull-out force: $p < 0.001$). Except for stiffness at 8 weeks, pull-out force, stiffness, and energy had increased significantly at 2, 4, and 8 weeks with up to a doubling in energy at 4 and 8 weeks (Table 9).

Long-term effect

Bisphosphonate-coated or control screws were inserted unilaterally into cortical bone in 21 rats and pulled out after 24 weeks (Table 10). For the bisphosphonate-coated screws, the pull-out force was increased by one quarter ($p = 0.05$; Table 11).

Discussion

This study confirms our previous finding of enhanced mechanical fixation of bisphosphonate-coated screws after 2 weeks (Tengvall et al. 2004). In addition, this difference in the mechanical fixation properties of bisphosphonate-coated screws and control screws was found to increase up to 8 weeks.

After our previous 2-week study, we erroneously considered it to be most likely that the posi-

tive effect of bisphosphonates was entirely due to the inhibition of the trauma-induced resorption that normally appears in the first few days after implantation. However, if this were the only effect, it should culminate within a week or two. Thus, the trauma effect cannot explain the continuously increasing difference in degree of mechanical fixation between bisphosphonate-coated screws and control screws. This long-term increase in mechanical pull-out of the bisphosphonates can only be explained by a long-lasting influence on the local balance between bone resorption and formation. At an injured site, or during *de novo* bone formation, osteoblasts and osteoclasts are not coupled as in normal background remodeling. Thus, a decrease in osteoclast activity can occur while osteoblasts are virtually unaffected. This will result in a net gain in local bone mass. In this way, the bisphosphonates can be said to have a local anabolic effect.

Pull-out strength in our model depends on the strength of the bone in the space between the screw threads, i.e. at a distance of half a millimetre or so from the implant surface. In order for the bone to become gradually stronger in our experiments, the bisphosphonates must have been present in this space to inhibit osteoclasts far away from the implant surface. This indicates that the bisphosphonates are re-cycled locally and re-used, and in this way work their way out from the implant surface. Bisphosphonates that are released from the bone by an osteoclast can attach to nearby bone mineral, and are obviously not destroyed inside the osteoclast, but survive intact (Lin 1996). Some of the bisphosphonates that are released might find their way into the circulation and be excreted, with a gradual reduction in the local amount as a result. The gradual migration of bisphosphonates away from the implant surface, which seems to be the only explanation for the increasing effect on strength, requires that some bisphosphonate-containing bone is in fact resorbed. Thus, it seems unlikely that the bone will remain totally unresorbed and eventually becomes fatigued (Rodan et al. 2004, Smith et al. 2004).

The above explanation for our findings remains hypothetical, but it should be possible to test it with radiolabeled bisphosphonate, for example. It is also supported by the finding of increased bone density at 0.2 mm distance from hydroxyapatite-

coated implants with an adherent bisphosphonate (Peter et al. 2005).

For historical and practical reasons, experiments on screw fixation in bone often use removal torque measurements. Removal torque is mostly related to friction between the implant surface and the bone, and is strongly influenced by surface texture, whereas we saw no difference between smooth and etched screws in our pull-out tests. Screws in clinical applications are almost never challenged by torque, but by forces that transfer the load to the bone between the bone threads. Thus, pull-out testing has greater clinical relevance, and the effects of surface texture can become over-emphasized in the interpretation of studies using torque measurements only. The effects of hydroxyapatite-coated cylindrical implants on pull-out force show that the density of bone in the 20 μm nearest to the implant is important for enhanced mechanical fixation (Peter et al. 2005).

In our study, about 10% of the rats had an abscess at the site of the screw. There were obviously shortcomings in the asepsis technique. Since then, we have corrected this and brought the abscess rate down. Interestingly, the infections did not influence the pull-out force. It appears that the infection was not able to spread from the subcutaneous space into the implant-bone interface. We have also seen this in some cases evaluated by histology.

Because of longitudinal bone growth, the screw inserted in cancellous bone will be positioned more distal to the physis with time, and hence surrounded by more cortical bone. This might explain the increase in pull-out force over time, at least for those screws that were inserted in cancellous bone. However, it does not explain the increase in the difference between control and bisphosphonate-coated screws over time.

The clinical applications of our coating technology are obvious. For example, dental implants in cancellous parts of the maxilla must remain load-protected for several months in order to let the local bone response produce enough bone for stability and load bearing. The effect of a bisphosphonate on net bone formation may shorten the time required for load protection. Also, with screws for fracture fixation in osteoporotic bone, bisphosphonate coating may maintain and improve fixation strength. Indeed, systemically administered

bisphosphonates have been shown to increase the degree of fixation of total joint replacements during the first postoperative year in patients (Hilding et al. 2000).

Implants such as screws or total joint replacements in cortical bone tend to lose some fixation strength postoperatively (Dhert et al. 1998). We were unable to demonstrate this effect in our cortical implants. Rat cortical bone has a different structure from that in larger animals, with a looser arrangement and no osteons. We believe, but have not shown, that postoperative resorption at the implant-bone interface in cortical bone can be blocked with bisphosphonate coating.

To conclude, bisphosphonate coating of stainless-steel screws enhances mechanical fixation when inserted into cancellous and cortical bone, with an increasing effect up to 8 weeks postoperatively.

Contributions of authors

KW: did most of the experimental work and also participated in planning, analysis and writing. PT: responsible for coating methods. PA: took the main part of planning, analysing and writing.

The authors thank Ali Sodeifi and Sara Arvidsson for technical assistance. This investigation was supported by the local strategic research project Materials in Medicine (Östergötland County Council/Linköping University), and the Swedish Research Council (project no. 2031).

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