Layer-by-layer gelatin/chitosan polyelectrolyte coated nanoparticles on Ti implants for prevention of implant-associated infections

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Abstract. Gelatin nanoparticles (Gb-NP) and layer-by-layer (LBL) coated NPs were developed to modify a Ti substrate surface to prevent implant-associated infections. Vancomycin (Van) was loaded into these materials to obtain GbV-NP and LBL-GbV-NP. The size of the GbV-NP (277.4 ± 1.4 nm) was smaller than that of LBL-GbV-NP (710.2 ± 4.6 nm) but both had a spherical shape. These coated materials showed no cytotoxicity and facilitated better cell proliferation by osteoblast-like cells compared to the bare Ti. This was probably due to the roughness of the coated NP that enhanced cell attachment to the surface. Both coated materials showed antibacterial activity against *S. aureus*. The release of Van from GbV-NP was higher from LBL-GbV-NP and this corresponded to their antibacterial activity. Furthermore the release profile of Van showed a sustained release. Thus both materials should be able to prolong the protection of implant-associated infections to the bone.

Keywords: nanomaterials, coatings, biocompatible polymers, vancomycin, Ti implantation

1. Introduction

Titanium (Ti) and Ti alloys have been extensively used in dental and orthopaedic areas as implants due to their biocompatibility, low density and good mechanical properties. However, unmodified Ti may host bacterial infections after implantation [1]. These infections often lead to implant failure, removal of implants, higher treatment costs, and also increased patient morbidity or in some cases perioperative mortality. Infections must be treated carefully with antimicrobial agents. High systemic doses of some agents may cause various side effects including nephrotoxicity and ototoxicity. Applications of local antimicrobial agents have generated much interest as an alternative drug delivery system. Basically, local drug delivery will allow for a better control of drug application directly at the required site/location for an extended period of time.

Various antibiotic coatings on Ti have been investigated to provide a local antibacterial delivery to a Ti implant [2]. Most studies have involved incorporating antibiotics into numerous types of materials coatings such as carbonated hydroxyapatite [3] chitosan (Chi) [4] and tetraethylorthosilane [5] either directly, as a film or a layer-by-layer (LBL) coating [6–8]. Modifications of titanium implant surfaces by covalent

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lently bonded antibiotics have also been employed [2, 9]. Recently nanoparticles (NPs) have been applied as an alternative coating on Ti implant surfaces [10–12]. These included the use of chitosan nanoparticle-based coating [10], such as antibiotic-loaded silk protein fibroin NPs and antibiotic-loaded electrospun coatings [12]. Nanostructure modifications of the Ti surface have several advantages. The increase of the Ti surface roughness by NPs has provided better cell attachment and enhanced the *in vitro* bone bonding capacity [11, 13, 14]. In addition, it was shown that nanostructured surfaces did reduce inflammation compared to a flat surface [15]. Therefore surface modifications of Ti can improve cell attachment and probably osseointegration by connecting between the Ti surface roughness of the NP coating and the newly formed bone tissue.

Coating by LBL is an advanced technique to assemble polyelectrolyte multilayer (PEM) films onto polymer surfaces, mesoporous silica nanoparticles and Ti surfaces by sequential adsorption of oppositely charged polyanions and polycations via electrostatic interactions [16–22]. This technique is expected to provide a more durable coating due to the electrostatic attractions between layers and between the first layer and substrate [6]. Several methods have been employed for LBL coating including centrifugation, microfluidics, dipping, casting and spraying [23, 24]. Gelatin is a hydrophilic, biodegradable protein derived from collagen by partially acidic (type A) or alkaline (type B, Gb) hydrolysis [25]. Chitosan is a polysaccharide comprised of glucosamine and N-acetylglucosamine. Both gelatin and chitosan have been extensively investigated and used in pharmaceutical applications due to their biocompatibility and biodegradable properties. Chitosan also has a feeble antibacterial activity [26, 27]. Recently, chi/gelatin as a polycation/polyanion for use as an LBL system has been successfully prepared [6]. This hydrophilic coating may provide an antiadhesive surface and reduce biofilm formation on the surface [28]. The electrostatic LBL self-assembly using Chi/Gb was thus employed in this study.

The objective of this study was to develop an effective antibiotic, vancomycin HCl (Van), loaded Gb-NP (GbV-NP) to produce the surface roughness that was subsequently coated with polyelectrolytes (Chi/Gb) using LBL assembly to prevent bacterial infection. This PEM coating NP (LBL-GbV-NP) could probably provide a sustained release of the Van. All

coating materials (Gb-NP, GbV-NP, LBL-GbV-NP) were characterized for their particle sizes, zeta potential and morphology. The interactions between Van and the NP were also investigated using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR). The amount of Van contained in the coating materials was analyzed. Cell proliferation and cytotoxicity were determined using osteoblast like cells (CAL-72). Antibacterial activity against *S. aureus* was also assessed. In addition the release of Van from the coating materials was examined.

2. Experimental

2.1. Chemicals

Gelatin type B (Gb, bovine bone type-B gelatin), Van, 25% glutaraldehyde, chitosan (medium molecular weight with a deacetylation degree >85%) and poly (ethylenimine) (PEI, $M_n = 65\,000$), were purchased from Sigma-Aldrich Chemical Co. St. Louis, Mo. Flat titanium thin films were deposited on borofloat glass substrates via electron-beam deposition. All films were deposited with a film thickness of 200 nm.

2.2. Preparation of nanoparticles

The Gb-NP and GbV-NP were prepared using a desolvation technique as previously described [29, 30]. A 20 mL aqueous solution of Gb (100 mg) with Van (20 mg) and without Van were prepared and adjusted to pH 6–10 by the addition of NaOH (0.1 M). Then the solution of Gb or GbV was stirred (600 rpm) at 50 °C until clear. To induce the desolvation process, acetone was added until a permanent faint turbidity was obtained. Subsequently, glutaraldehyde solution (8% w/v, 250 μ L) was added to harden the particles. The preparation was then stirred overnight at 1000 rpm. The obtained NPs (Gb-NP and GbV-NP) were separated from the solution by centrifugation at 9000 rpm for 20 min, then washed 2–3 times with distilled water, re-suspended and dispersed in 20 mL of distilled water for use. The optimal pH based on the zeta potential and size of the obtained GbV-NP

Table 1. Hydrodynamic diameters, polydispersity index and zeta-potential of GbV-NP prepared at various pHs ($n = 3$, mean $\pm$ SD)

pH	Diameter [nm]	Polydispersity index	Zeta-potential [mV]
6	410.5 $\pm$ 3.8	0.222 $\pm$ 0.015	–19.7 $\pm$ 0.4
8	277.6 $\pm$ 1.2	0.145 $\pm$ 0.015	–27.1 $\pm$ 0.2
10	280.7 $\pm$ 2.4	0.177 $\pm$ 0.015	–26.7 $\pm$ 0.3

was pH 8 (Table 1) and this pH was employed for the NP preparation.

2.3. Preparation of PEM shell surrounding nanoparticles

The PEM shell coating on GbV-NP was carried out by coating the first layer with Chi and the second layer with Gb. Chitosan (60 mg) was dissolved in 0.2 M acetic acid (30 mL) to obtain the Chi concentration of 2 mg/mL. The GbV-NPs (100 mg) were then suspended in the Chi solution (30 mL) and stirred for 15 min at room temperature. Subsequently, the mixtures were centrifuged and washed with distilled water to obtain the GbV-NP/Chi coating. This procedure was also employed for coating the GbV-NP/Chi with Gb by using the Gb solution (2 mg/mL) to obtain GbV-NP/Chi/Gb or LBL-GbV-NP.

2.4. Preparation of nanoparticles coating on Ti surface

The deposited Ti films were immersed in PEI solution (5 mg/mL) for 20 min. This created a precursor layer with a stable positive charge to initiate the electrostatic force with Gb-NP, GbV-NP or LBL-GbV-NP that had a negative charge. These NPs were deposited onto the Ti/PEI sheets by drop casting as previously described [10]. Briefly, 200 μ L of NP suspension was dropped onto the Ti/PEI and subsequently the coatings on the Ti/PEI sheets were oven-dried at 37 °C to obtain Ti/Gb-NP, Ti/GbV-NP and Ti/LBL-GbV-NP.

2.5. Characterization of nanoparticles

The suspension of NP (100 μ L) was dispersed in 2 mL of deionized water and hydrodynamic size measurements were performed using dynamic light scattering (DLS) on a Nano-ZS90 Zetasizer (Malvern Instruments Ltd. Co., UK). Zeta (ζ) potentials were measured also using a Nano-ZS90 Zetasizer in capillary cells. All the measurements were conducted at room temperature in triplicate.

The morphology of the oven-dried GbV-NP and LBL-GbV-NP on the Ti surface was characterized by SEM (S440i, Leica, Germany) at a magnification of $\sim 5000\times$ operated at 20 kV. A Focused Ion Beam Scanning Electron Microscope (FIB-SEM, AURIGA 60 CrossBeam[®], Carl-Zeiss AG, Oberkochen, Germany) was also employed for morphological characterization and cell proliferation on the samples.

The possible interactions between the Van and the utilized polymers were investigated using Fourier transform infrared (FTIR) spectroscopy (FTIR spectrometer Alpha-P with evaluation software Opus, Bruker, Rheinstetten, Germany). Samples included Van and Gb powders and freeze dried samples of Gb-NP, GbV-NP and LBL-GbV-NP. The FTIR spectra were recorded (range: 375–4000 cm^{-1} , resolution: 4 cm^{-1} , number of scans: 24) in an attenuated total reflection (ATR) mode.

2.6. Determination of vancomycin entrapment in gelatin nanoparticles

The entrapment efficiency (EE) of Van in GbV-NP was determined by the indirect method as previously described [10]. The GbV-NP were separated by centrifugation of the colloidal suspension for 20 min at 9000 rpm at 25 °C (section 2.2), and the free Van (Van_{free}) in the supernatant was measured by UV-Vis spectrophotometry at 282 nm as previously described [31]. The %EE was calculated according to Equation (1):

$$\%EE = \frac{\text{Van}_{\text{tot}} - \text{Van}_{\text{free}}}{\text{Van}_{\text{tot}}} \cdot 100 \quad (1)$$

where Van_{tot} denoted the amounts of Van present in the solution.

2.7. Vancomycin release study

In vitro release studies of Van were carried out on the GbV-NP and LBL-GbV-NP coating on Ti/PEI. The coated materials were immersed in 1.5 mL of PBS (pH 7.4) in a 24 well plate at 37 °C in the oven as previously described [11, 12]. At pre-determined time intervals, an aliquot (500 μ L) was withdrawn and the amount of Van was determined by UV spectroscopy at 282 nm as mentioned in section 2.6. A total volume of 1.5 mL was maintained by refilling with 500 μ L of the same medium after each withdrawal. All experiments were performed in triplicate.

2.8. Antimicrobial test

Antimicrobial activity of the coated samples was investigated based on an agar diffusion test (Kirby-Bauer antibiotic test [32] and *Staphylococcus aureus* MRSA 134/94 (methicillin-resistant strain) as a model organism.

S. aureus was pre-cultured for 12 h in LB (lysogeny broth) medium. Afterwards, the bacterial culture was

diluted using physiological NaCl solution to obtain an optical density of 0.5 which according to the McFarland standard roughly corresponds to $1.5 \cdot 10^8$ CFU (colony forming units) per mL. For preparation of the agar diffusion plate, 100 μ L of the bacterial suspension was added to 34 mL of molten medium-agar solution (at 56 °C) per plate. After cooling, the coated samples were placed upside down on the agar diffusion plates. The plates were incubated for 24 h at 37 °C. The experiments were performed in triplicate.

2.9. Cell proliferation, cell viability test and scanning electron microscopy

Cell proliferation on the coated samples was tested using osteoblast like cells (CAL-72, derived from human osteosarcoma ACC439; German Collection of Microorganisms and Cell Cultures DSMZ, Braunschweig, Germany). The cells were cultured in Dulbecco's modified eagles medium (DMEM, VWR International, Dresden Germany) containing 10% fetal bovine serum, at 37 °C in a humidified 5% CO₂ atmosphere. The coated materials ($n = 5$ replications) were placed in tissue culture poly(styrene) well plates and incubated with 10 000 cells per cm² (corresponding to 20 500 cells per well) for 24 and 72 h (3 days) at 37 °C in an humidified 5% CO₂ atmosphere. After incubation, the samples were gently washed twice with phosphate buffered saline (PBS) to remove non-adherent cells, trypsinated with 0.25% trypsin in PBS, and subsequently counted with a Z1 Coulter counter (Beckman Coulter GmbH, Krefeld, Germany). A plate well without sample was used as a control. Cell viability was determined by the MTT assay. After culturing the cells for 3 days at 37 °C, 5% CO₂, the medium was then removed; 200 μ L of methylthiazolyldiphenyl-tetrazolium bromide (MTT, Aldrich 135038, Sigma-Aldrich) solution (5 mg/mL) and 1.8 mL PBS were added to each well and incubated at 37 °C for a further 4 h. After discarding the MTT solution, the dark blue formazan crystals were dissolved by adding 100 μ L of dimethyl sulfoxide (DMSO) and quantified using a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at 570 nm. The results are reported as a percentage in comparison with a cell culture medium that was used as a control.

Osteoblast cells cultured on Ti/Gb-NP, Ti/GbV-NP and Ti/LBL-GbV-NP were evaluated by FIB-SEM after being cultured for 3 days. For the FIB-SEM im-

aging, samples with adherent cells were gently washed twice with PBS to remove non-adherent cells and, afterward, fixed in glutaraldehyde solution (2.5%) for 24 h at room temperature. After fixation, samples were washed for 30 min in PBS buffer solution and twice in distilled water. Subsequently, samples were dehydrated using an ascending ethanol series from 10 to >99.9 % with 30 min incubation per step and two times at 60 min for the last step. The dehydrated samples were dried by the critical point method (EMITECH K850, Quorum Technologies Ltd, East Grinstead, UK) and gold sputter coated (S150B, Edwards Ltd, Crawley, UK).

All data were expressed as mean values $\pm$ standard deviation. Statistical analysis was performed using the IBM SPSS Statistics version 22 for Windows. Data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey post hoc test for multiple comparison tests.

3. Results and discussion

3.1. Preparation and characterization

As listed in Table 2, the hydrodynamic diameter of the Gb-NP was found to be 216.3 nm with a polydispersity index (PDI) of 0.052 and a zeta potential of -29.9 mV which were similar to previous reports [29, 33]. The PDI values were around 0.14 for the GbV-NP, GbV-NP/Chi and LBL-GbV-NP. In the same way as previous studies, the size and zeta potential values of the gelatin NPs were dependent on various parameters including temperature, pH and the concentration of glutaraldehyde [30, 33]. In this study, the glutaraldehyde concentration and temperature for preparing the NPs were the optimal concentration and temperature reported in the literature [30, 33]. The size of the drug loading Gb-NP (GbV-NP) was slightly increased to 277.4 nm compared to the unloaded NP. The Chi (polycation) coating onto the GbV-NP (negative charge) resulted in a larger size (324 nm) and the zeta potential was changed to a positive charge ($+27.2$ mV). The GbV-NP/Chi was converted to a

Table 2. Hydrodynamic diameters, polydispersity index and zeta-potential of various nanoparticulates prepared in three separate experiments (mean $\pm$ SD)

Samples	Diameter [nm]	Polydispersity index	Zeta-potential [mV]
Gb-NP	216.3 $\pm$ 1.6	0.052 $\pm$ 0.033	-29.9 ± 0.6
GbV-NP	277.4 $\pm$ 1.4	0.144 $\pm$ 0.018	-27.0 ± 0.3
GbV-NP/Chi	324.8 $\pm$ 1.0	0.144 $\pm$ 0.017	$+27.2 \pm 0.8$
GbV-NP/(Chi/Gb) or LBL-GbV-NP	710.2 $\pm$ 4.6	0.140 $\pm$ 0.035	-20.0 ± 0.2

Figure 1. SEM micrographs of GbV-NP (a) and LBL-GbV-NP (b)

Figure 2. FIB-SEM characterization of Gb-NP (a), GbV-NP (b) and LBL-GbV-NP (c)

negative charge (−20 mV) again after being surrounded with Gb (polyanion). This confirmed that the assembly had taken place on the surface of the NPs. As shown in Figures 1 and 2, all the NPs (Gb-NP, GbV-NP and LBL-GbV-NP) had spherical shapes with a smooth surface. The Gb-NP, GbV-NP and

LBL-GbV-NP showed the same morphology but were of a slightly larger size after coating with the Chi/Gb (LBL-GbV-NP). The Van and Chi/Gb coating did not cause any observed effect on the surface of the NPs. The NPs size ranges were in agreement with the mean particle size characterized by DLS.

3.2. ATR FTIR analysis

Figure 3 shows the ATR-FTIR spectra of the Van, Gb, Gb-NP, GbV-NP and LBL-GbV-NP. The ATR-FTIR spectra of all samples showed broad OH bands of a phenolic or acid group from the Van and Gb at about 3200 cm^{-1} . Due to their broad bands, the interactions at these functional groups of each sample could not be detected. The C=O stretching bands of the Van and Gb were observed at 1647 and 1633 cm^{-1} , respectively. The C=O stretching of Gb-NP, GbV-NP and LBL-GbV-NP appeared, respectively, at 1639 , 1638 and 1637 cm^{-1} , which were at a relatively lower wave number than Van. The spectra of all the NPs were comparable to that of Gb since Gb was the major component in the NPs. The slight difference of the NPs and Gb may be due to the cross-linked Gb caused by the glutaraldehyde in the NP preparation. The prominent bands of the Van powder were unfortunately not detected in the spectra of the NPs containing Van, GbV-NP or LBL-GbV-NP. This may be due to the Van loading to only 11.6% in the NPs, based on the NP preparation and the %EE of Van. Thus, the spectrum of the Van in the NPs was probably shielded by the Gb spectrum. Therefore, it may be difficult to conclude whether Van did interact with the Gb-NP. Nevertheless, the covalently attached Van to the Ti implant surfaces has previously been report-

Figure 3. ATR-FTIR of vancomycin (Van) and gelatin B (Gb) powders and various types of nanoparticles

ed to be able to kill *S. aureus* after its cleavage from the Ti surfaces [34]. In this study, if Van could interact with Gb-NP, it would be by non-covalent interactions and could be easily dissociated from the Ti surface since Van could be detected in the EE experiment and the release studies (section 3.3). Thus, the antibacterial activity of Van in the materials was retained.

3.3. Drug release study

The %EE of Van in the GbV-NP as determined using Equation (1) was 70%. Figure 4 shows the release profile of Van from the GbV-NP and LBL-GbV-NP coating on the Ti substrate. The release of Van from Ti/GbV-NP was up to $120\text{ }\mu\text{g}$ but that from the Ti/LBL-GbV-NP was about $30\text{ }\mu\text{g}$ in 48 h. This was probably due to the polyelectrolyte coating layer that was able to hinder the release of the drug. The release of Van from both the NPs demonstrated a sustained release of the drug and would be able to prolong the protection of the bone from infection.

3.4. Antibacterial efficacy

The antibacterial efficiency of the Ti/GbV-NP and Ti/LBL-GbV-NP against *S. aureus* which is the most common micro-organisms that causes implant-associated infections was determined [12, 34, 35]. As shown in Figure 5, clear zones were not detected for the bare Ti or the Ti with the Gb-NP coating. Hence these two materials had no antibacterial activity. Clear zones were observed from both the materials coated with Van (Ti/GbV-NP and Ti/LBL-GbV-NP). The inhibition zones that were detected after incubation for 24 h of Ti/GbV-NP was slightly larger than those for the Ti/LBL-GbV-NP. This was consistent with the release of Van from the coated materials and demon-

Figure 4. Release profiles of vancomycin from GbV-NP and LBL-GbV-NP coated on Ti substrate

Figure 5. Inhibition zone caused by Van (30 μ g) (1), bare Ti (2) and various nanoparticle, Gb-NP (3). GbV-NP (4), LBL-GbV-NP (5), coatings on Ti substrate. Based on NP preparation and %EE, approximate 140 μ g of Van was loaded on the in GbV-NP and LBL-GbV-NP.

strated that both materials would effectively reduce bacterial infection.

3.5. Cell proliferation and cell viability

Osteoblast cells have been widely used as a model to investigate the behavior of osteoblasts on polymer surfaces [36]. In this study, cell proliferation of osteoblast like cells (CAL-72) on the bare Ti and coated materials is shown in Figure 6. Cell proliferation on the Ti/LBL-GbV-NP ($5.6 \cdot 10^4$ cells) was slightly

Figure 6. Proliferation of osteoblasts cultured on Ti substrate and various nanoparticle coatings on Ti substrate for 3 days (mean $\pm$ SD, $n = 5$ pergroup)

higher than that on the Ti/GbV-NP ($5.5 \cdot 10^4$ cells) but not significantly different ($p > 0.2$). However cell proliferation on both the materials was higher than that on the Ti/Gb-NP ($p < 0.001$). Cell proliferation on the bare Ti was not statistically different from that on the control ($p > 0.9$) and on the Ti/Gb-NP ($p > 0.6$).

As shown in Figure 7, the viability of the osteoblast cells on all the test samples with a culture period of 3 days showed favorable cell viability on all coated Ti: Ti/Gb-NP ($105 \pm 4.1\%$), Ti/GbV-NP ($108 \pm 2.9\%$) and Ti/LBL-GbV-NP ($110 \pm 3.9\%$). Cell viability on the coated materials was all very similar ($p > 0.3$). Cell viability on Ti/GbV-NP and Ti/LBL-GbV-NP was higher than that on the bare Ti ($p < 0.004$) ($100 \pm 2.9\%$) which was not significantly different from the control ($p > 0.7$). This cell viability result demonstrated that these coated materials were not toxic to the osteoblast like cells. In addition, the coated materials did induce more cell proliferation than the control.

The adhesion and spread of the osteoblasts over the biomaterial was the initial step of cell-material interaction and this affected the subsequent cell proliferation and/or differentiation [6]. As shown in Figure 8, adhered cells developed more on the coated materials than on the bare Ti. This demonstrated a preferable interaction between the cells and the coated materials. Furthermore, LBL-GbV-NP coated onto the Ti showed the most attachments by the cells. Consequently, cell proliferation on Ti/LBL-GbV-NP was higher than that on the Ti/GbV-NP and the Ti/Gb-NP.

Figure 7. Cell viability of osteoblasts seeded onto Ti substrate and various nanoparticle coatings on Ti substrate for 3 days (mean $\pm$ SD, $n = 5$ pergroup)

Figure 8. FIB-SEM images of osteoblast like cells on bare Ti (a) and on Ti coated with Gb-NP (b), GbV-NP (c) and LBL-GbV-NP (d) after cultured for 3 days

4. Conclusions

Modifications to the titanium surface by coating with GbV-NP and LBL-GbV-NP were prepared in order to prevent implant-associated infections. The coated materials were not cytotoxic. However, the Ti coated materials showed a significantly higher proliferation of osteoblast like cells than the bare Ti. The improvement of cell proliferation was probably due to the roughness of the NP coatings that enhanced cell attachment to the surface. The antibacterial activity of the Ti/GbV-NP was slightly higher than that of Ti/LBL-GbV-NP which agreed with the data on the release of Van from these coating materials. Moreover, the coated materials demonstrated a sustained release of Van. Thus the coated materials loaded with the drug may prevent infections for a longer period of time.

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