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Kevin's paper received a *Quaesitum* outstanding paper award.

Kevin Patel

Evaluation of Two Different Neutralization Methods for
Chitosan Coatings

Faculty Sponsor

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Abstract

Chitosan has been investigated as a coating for musculoskeletal implants due to its osteoconductive and local drug delivery properties. Since chitosan is dissolved in dilute organic acid, the residual acid components must be removed post-coating, which is typically done using alkali and/or ethanol treatments. Neutralization affects coating surface chemistry, adhesion, swelling, and other properties. The aim of this study was to compare the effects of NaOH neutralization versus phosphate buffer neutralization on solution cast coatings on commercially pure titanium. Properties evaluated were contact angle, swelling/drug release, coating adhesion, cell attachment, and cell proliferation. Results show that phosphate buffer-neutralization produces coatings with properties that are more favorable for implant device applications.

Introduction

Chitosan, a versatile biopolymer derived from chitin, has been investigated as a coating for musculoskeletal and dental implants due to its osteoconductive and local drug or growth factor delivery capabilities [1, 2]. To produce chitosan coatings, chitosan is typically dissolved in dilute organic acid, which protonates the amino groups of the molecules. Following substrate coating, the residual acid components must be removed to prevent harmful acidic salts from leaching out [3].

Neutralization of the residual acidic components is typically completed with alkali and/or ethanol treatments. Many different methods of neutralization have been used in the literature; however, the neutralization method can affect coating properties such as surface chemistry, adhesion, swelling, and many others [4-6]. Coating properties that are favorable for musculoskeletal and dental implant devices include good coating adhesion strength to be able to withstand the implantation process and mechanical forces when in use. The coating must also be able to show good cell attachment and growth to promote osseointegration of the implant device. In addition, since chitosan coatings are extensively investigated for delivery of local therapeutic agents, the coatings must also be able to swell and absorb these agents and passively release them over an extended period of time [7-9].

It was previously shown that the adhesion strength of chitosan coatings can be approximately tripled when bonded to cp titanium via silane reactions [10]. This involves bonding chitosan molecules to cp titanium via silane molecules. Consequently, the method used to neutralize the coating must not be detrimental to these silane bonds.

In this study, the most common neutralization method, NaOH, was compared to neutralization using a potassium phosphate buffer, which is a less common method for neutralizing chitosan coatings [7]. Properties evaluated included contact angle, swelling, protein release, coating adhesion, cell attachment, and cell growth. The Saos-2 osteosarcoma cell line (obtained from American Type Culture Collection, Manassas, VA) was used in this investigation, as this is a well characterized human cell line that presents similar proliferation and mineralization to osteoblastic cells [11]. Due to the lack of reliance on extreme pH for neutralization, it was hypothesized that phosphate buffer neutralization would result in coating properties that were more favorable for implant device applications.

Methods and Materials

Coating Preparation

Commercially pure (cp) titanium coupons (ASTM F67) were wet sanded and polished with silicon carbide paper up to 1200 grit followed by ultrasonic cleaning in acetone, ethanol, and deionized water for 10 minutes each. Samples were passivated in 30% nitric acid for 30 minutes. Chitosan (95% deacetylated, Heppe Medical, Halle, Germany, molecular weight 300 – 700 kDa) at 2 wt% in 1% acetic acid was bonded to cp titanium samples via silanation reactions. In summary, samples hydrated in deionized water for 24 hours were placed in 95:5 vol% ethanol/water solution acidified with 10 M acetic acid to pH 4.5. Triethoxysilylbutyraldehyde (Gelest, Morrisville, PA) was added such that the final concentration of the silane was 2%. The pH was maintained between 4.5 and 5.5 for 10 minutes with 10 M acetic acid or 1 M NaOH. Samples were washed with ethanol and cured at 110°C for 10 minutes, followed by solution casting with the chitosan solution at 0.5 mL/cm². Samples were allowed to air dry at room temperature for 6 to 7 days [10]. Dry samples were neutralized in either 0.5 M NaOH or 0.25 M potassium phosphate buffer, pH 7, by submersion for 30 minutes followed by rinsing twice with deionized water.

Evaluation of Physical Properties

Water contact angles were determined using a VCA Optima (AST Products, Inc., Billerica, MA). These measurements were used to evaluate the hydrophilicity of samples (n=6, approximately 1 cm × 1 cm × 0.1 cm).

Swelling studies were performed by measuring the mass of samples (n=6, approximately 1 cm × 1 cm × 0.1 cm) that had been previously desiccated for 24 hours. Samples were submerged in phosphate buffered saline (PBS) for 24 hours. The mass of samples after submersion were determined after gently tapping off excess PBS. Swelling ratios were determined by calculating the ratio of change in mass to dry mass.

Coating adhesion tensile strengths were determined by a method that was previously described in the literature [10]. Three aluminum pins (9.5 mm diameter) were adhered to coated samples (n=5, approximately 2 cm × 5 cm × 0.1 cm) with Loctite 454 gel adhesive. A 5 kg uniform load was applied immediately after bonding surfaces and left for 24 hours at room temperature to ensure an even layer of adhesive. An Instron 4465 (Houston, TX) was used to pull pins at a rate of 10 mm/min. The setup is

shown in Figure 1. Pins glued to bare cp titanium samples were also tested to determine the strength of the adhesive.

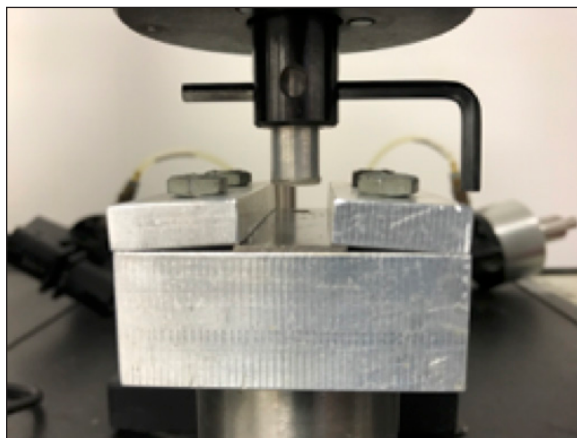


Figure 1. Setup used to measure tensile adhesion strength of coatings

Protein Elution

Samples ($n=3$, approximately $1\text{ cm} \times 1\text{ cm} \times 0.1\text{ cm}$) were passively loaded with $100\text{ }\mu\text{g}$ chymotrypsin in a buffer solution of 5 mM glutamic acid, 5 mM NaCl, 2.5% glycine, 0.5% sucrose, and 0.01% Tween 80 at pH 4.5. This was done by pipetting the loading solution onto coatings and allowing the solution to swell completely into the coating at 4°C . Chymotrypsin was used since it is an analogue of bone morphogenetic protein-2 (BMP-2), which is a growth factor that has seen many clinical uses in orthopedic and dental applications [12, 13]. Samples were eluted in 1 mL PBS in a 37°C incubator for 6 days. Eluates were collected every 24 hours and replaced with fresh PBS. Protein concentrations of the eluates were determined using the Coomassie Plus protein assay (Thermo Fisher Scientific, Waltham, MA).

Cell Attachment and Proliferation

Cell attachment to coatings was evaluated by seeding circular samples ($n=6$, approximately 1.56 cm diameter $\times 0.1\text{ cm}$) placed at the bottom of a 24-well cell culture plate. Fifty-thousand Saos-2 cells were seeded and after 4 hours, the number of cells remaining in the cell culture medium was determined with the use of a Scepter cell counter (Merck Millipore, Darmstadt, Germany). The number of cells remaining in the medium was

subtracted from the original number of cells seeded to determine the number of cells attached to the coatings. Bare cp titanium samples were used as control. Samples were also fluorescently stained with Alexa Fluor 488 dye (Thermo Fisher Scientific, Waltham, MA) and NucBlue stain (Thermo Fisher Scientific, Waltham, MA) for visualization.

Cell proliferation was evaluated by seeding 10,000 Saos-2 cells on samples ($n=4$, approximately 1.56 cm diameter \times 0.1 cm) at the bottom of a 24 well cell culture plate. Cell number was quantified at days 1, 3, 5, 7, and 9 with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Fitchburg, WI). All plates were seeded at day 0 and standards curves were produced at days 1, 3, 5, 7, and 9.

Statistical Analyses

Analysis of variance (ANOVA) with Tukey post-hoc tests were used for statistical analysis of contact angle, coating adhesion, protein elution, cell attachment, and cell proliferation data. T-test was used for swelling data. A p-value less than 0.05 was considered significant for all analyses.

Results

Physical Properties

Contact angle, swelling, and coating adhesion data are shown in Table 1. Contact angle data show that coatings neutralized in NaOH were significantly more hydrophilic than bare cp titanium ($p=0.029$), but no significant difference was determined between the phosphate buffer-neutralized coatings and the other two groups. Swelling ratios showed that coatings neutralized in a phosphate buffer swelled to a greater degree than coatings neutralized in NaOH, and this difference was statistically significant ($p=0.0009$). Tensile adhesion strength values suggest that phosphate buffer-neutralized coatings were also characterized by a significantly greater adhesion strength than coatings neutralized in NaOH ($p=4.3 \times 10^{-11}$). Since the adhesion strength of the adhesive on bare cp titanium was significantly greater than the measured strengths of both coatings, it can be assumed that the adhesive did not dissolve through the coatings and adhere to the underlying cp titanium.

Substrate	Contact Angle, n=6 (Degrees)	Swelling Ratio, n=6 (%)	Adhesion Strength, n=5 (MPa)
NaOH neutralized chitosan	75.6 ± 4.4 ^a	1.2 ± 0.2 ^a	1.6 ± 0.1 ^a
Phosphate buffer neutralized chitosan	84.7 ± 14.4 ^{ab}	1.8 ± 0.1 ^b	1.8 ± 0.1 ^b
cp titanium	91.7 ± 7.4 ^b	N/A	2.5 ± 0.1 ^c

Table 1. Summary of contact angle, swelling, and adhesion strength data (Superscripts indicate statistical significance, data in each column with similar letter superscripts are statistically similar)

Protein Elution

Figure 2 shows the in vitro release profile from samples passively loaded with 100 µg chymotrypsin. Both coatings resulted in burst protein release; however, coatings neutralized in a phosphate buffer were able to extend protein release until approximately day 6 as compared to the NaOH neutralized coatings which only extended release until day 3. Statistical significance was determined at each time interval. Statistically significant differences in chymotrypsin release between the two coatings were determined for days 2 to 4, during which the amount of chymotrypsin release from the phosphate buffer-neutralized coatings was greater than the NaOH-neutralized coatings ($p < 0.5$). Bare cp titanium samples released almost all loaded protein in the first day as expected.

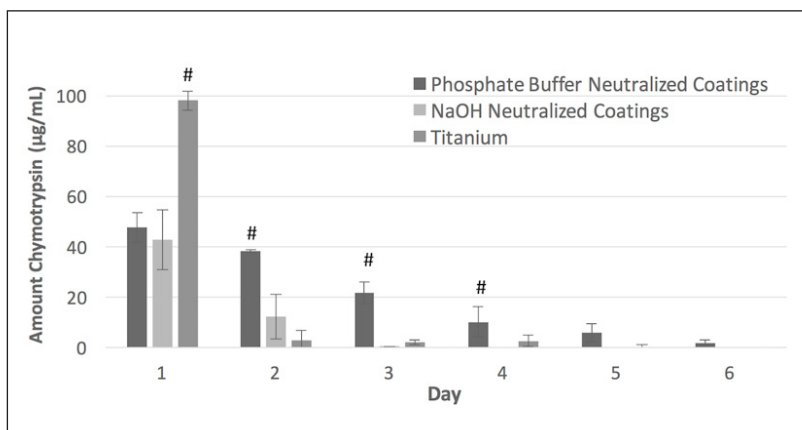


Figure 2. Chymotrypsin elution from samples (# denotes a statistically significant difference from all other groups at that time point), n=3

Cell Attachment and Proliferation

Table 2 shows the number of attached cells 4 hours after seeding samples with 50,000 Saos-2 cells. The data shows that only the number of cells attached to the NaOH-neutralized samples was statistically different ($p=0.025$). Figure 3 shows the attached cells stained with fluorescent dye. Cells exhibited good spreading for all groups.

Substrate	# Cells Attached
NaOH neutralized coatings	$37,000 \pm 3,180$
Phosphate buffer neutralized coatings	$41,800 \pm 3,130$
cp titanium	$43,500 \pm 3,690$

Table 2. Summary of Saos-2 cell attachment data, $n=6$

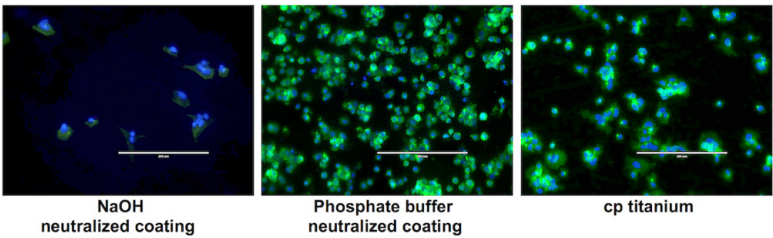


Figure 3. Cell attachment on coatings and cp titanium, 4X magnification

Figure 4 shows the growth of Saos-2 cells on samples. In general, both coatings exhibited similar cell growth during the 9-day period, and both coatings generally showed lower cell count than the cp titanium and cell culture plate controls. No statistically significant difference was determined between the cell count on coatings neutralized in a phosphate buffer and NaOH with the exception of days 1 and 9. Coatings neutralized in a phosphate buffer contained fewer cells than NaOH neutralized coatings at days 1 and 9.

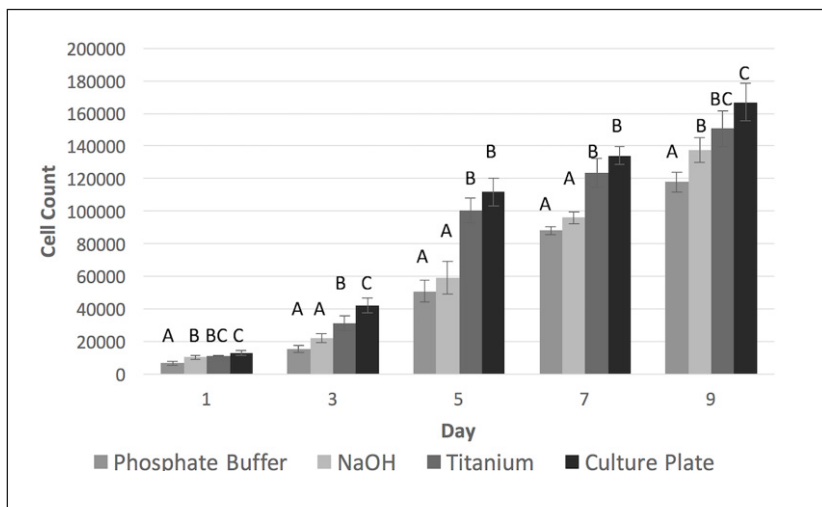


Figure 4. Saos-2 cell growth on samples (letters above data denote statistical significance), n=4

Discussion

The aim of this study was to compare chitosan coatings neutralized in NaOH or a phosphate buffer for use with musculoskeletal or dental implants. Coatings were produced with silane bonding reactions to obtain greater adhesion strength. The coatings produced in this study appeared a slightly yellow-amber color, which is associated with the formation of imine bonds between the silane and chitosan molecules [10].

Physical properties of the resulting coating are of great importance as these will predict the performance of the coating when used with an implant. The important functions required of a musculoskeletal or dental implant coating include good adhesion strength and osteoconductivity. Chitosan coatings have also been extensively investigated as a means for local drug or growth factor delivery, thus these coatings must also exhibit good swelling and elution profile of therapeutic agents.

There was initial hesitancy due to the adhesive used to adhere aluminum pins to coatings possibly dissolving the coating and adhering to the underlying cp titanium instead. However, since the bond strength of the pins glued to bare cp titanium was greater than that of the pins glued to coatings, it can be assumed that the glue did not dissolve through the

coatings. The tensile adhesion strength of the phosphate buffer-neutralized coating was greater than that of the NaOH-neutralized coating by approximately 0.2 MPa. Both of the strength values were similar to the previously reported value [10]. However, as also reported previously, these values are lower than the 6.7 – 26 MPa bond strengths reported for common plasma sprayed hydroxyapatite coating [14]. Thus, further work is needed to increase the overall bond strength of the coating. However, these results suggest that phosphate buffer neutralization produces coatings with greater bond strength. The greater bond strength is speculated to result from the reliance on less extreme pH for neutralization. As explained in the literature, high pH results in bond breaking of the silane-titanium bond [15]. Thus, the NaOH may have disrupted these silane bonds resulting in lower coating adhesion strength.

Water contact angle data are important to predict cell adhesion as more hydrophilic surfaces are better able to support protein adherence, which is the means by which cells adhere to implant surfaces. The data shows that the NaOH neutralized coatings were more hydrophilic than both phosphate buffer neutralized coatings. This suggests that cell adhesion on NaOH-neutralized coatings should be best. However, cell adhesion data contradicts this, which shows that the phosphate buffer-neutralized coatings exhibited more attached cells. This can be rationalized by the fact that NaOH neutralization deprotonates the chitosan amine, while phosphate buffer neutralization is speculated to not cause this to the same extent. Thus, the greater positive charge of the more protonated chitosan coating is able to support greater protein and cell adhesion.

The swelling ratio of phosphate buffer-neutralized coatings was also shown to be greater, which suggested that it would be better for local therapeutic agent delivery by passive loading. This was shown to be the case when samples were passively loaded with chymotrypsin to model the release of BMP-2. The phosphate buffer-neutralized coatings were able to extend the release by at least 3 additional days compared to that of the NaOH neutralized coatings. Again, this can be rationalized by the speculation that phosphate buffer neutralization causes less deprotonation of chitosan amines leading to a greater overall attraction of protein molecules due to molecular charge differences. However, the elution data shows a loss of approximately half the loaded protein in the NaOH group. This may have occurred due to NaOH remaining in the coating following

neutralization and causing pH-dependent protein breakdown. Many osteogenic growth factors require a longer release profile, since osseointegration occurs over a longer time period [16]. The initial burst release shown by these two coatings may reduce the amount of growth factor available at later times to promote osseointegration. However, there is work in the literature pertaining to extending local therapeutic agent delivery by coupling chitosan with other substances [17]. In these cases, the use of phosphate buffer-neutralization may help to extend the release profiles of these agents.

Preliminary bone cell growth data shows that there was little difference between the growth of these cells on coatings treated by the two neutralization methods. Only days 1 and 9 showed a statistically significant difference in which the phosphate buffer-neutralized coatings contained slightly fewer cells. Thus, both neutralization methods maintain the osteoconductivity of the chitosan. Further work must be completed to investigate cell growth over a longer time span, cell differentiation, mineralization, and in vivo implant success need to be completed to supplement these initial cell growth data.

Conclusion

Chitosan coatings have been extensively investigated to take advantage of their osteoconductive and local therapeutic agent delivery properties. Following the coating process, neutralization of acidic components must be completed before in vivo use. The method used to neutralize coatings affects the properties of the resulting coating. The aim of this study was to compare the effects of NaOH-neutralization versus phosphate buffer-neutralization on solution cast coatings on cp titanium. Contact angle, adhesion strength, swelling, protein elution, cell attachment, and cell proliferation data suggest that phosphate buffer-neutralization produces coatings that exhibit better properties for use in musculoskeletal and/or dental implant device applications.

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