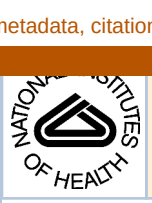

Published in final edited form as:

Biomaterials. 2008 October ; 29(28): 3882–3887. doi:10.1016/j.biomaterials.2008.06.007.

The effect of surface demineralization of cortical bone allograft on the properties of recombinant adeno-associated virus coatings

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Abstract

Freeze-dried recombinant adeno-associated virus (rAAV) coated structural allografts have emerged as an approach to engender necrotic cortical bone with host factors that will persist for weeks following surgery to facilitate revascularization, osteointegration, and remodeling. However, one major limitation is the nonporous cortical surface that prohibits uniform distribution of the rAAV coating prior to freeze-drying. To overcome this we have developed a demineralization method to increase surface absorbance while retaining the structural integrity of the allograft. Demineralized bone wafers (DBW) made from human femoral allograft rings demonstrated a significant 21.1 % (73.6 ± 3.9 % vs. 52.5 ± 2.6 %; $p<0.001$) increase in percent surface area coating versus mineralized controls. Co-incubation of rAAV-luciferase (rAAV-Luc) coated DBW with a monolayer of C3H10T1/2 cells in culture led to peak luciferase levels that were not significantly different from soluble rAAV-Luc controls ($p>0.05$), although the peaks occurred at 60hrs and 12hrs, respectively. To assess the transduction efficiency of rAAV-Luc coated DBW *in vivo*, we first performed a dose response with allografts containing 10^7, 10^9 or 10^{10} particles that were surgically implanted into the quadriceps of mice, and assayed by *in vivo* bioluminescence imaging (BLI) on days 1, 3, 5, 7, 10, 14, and 21. The results demonstrated a dose response in which the DBW coated with 10^{10} rAAV-Luc particles achieved peak gene expression levels on day 3, which persisted until day 21, and was significantly greater than the 10^7 dose throughout this time period ($p<0.01$). A direct comparison of mineralized versus DBW coated with 10^{10} rAAV-Luc particles failed to demonstrate any significant differences in transduction kinetics or efficiency *in vivo*. Thus, surface demineralization of human cortical bone allograft increase its absorbance for uniform rAAV coating, without affecting vector transduction efficiency.

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Keywords

Allograft; Bone; Recombinant Adeno-Associated Virus (rAAV); Freeze-dried; Bioluminescence Imaging (BLI)

Introduction

Although massive allografts are widely used during reconstructive surgery of critical defects in long bones, their limited osteogenic and remodeling potential is directly associated with the 25% to 35% failure rate within 3-years due to fracture and nonunion [1,2]. For those massive allografts that survive, the failure rate at 10-years has been documented to be as high as 60% [3-5]. The fractures at this late stage are the result of the accumulation of microcracks that cannot be repaired by the necrotic bone. As a result of this poor clinical success, the use of structural allografts has been restricted to repair segmental defects following tumor resection in cancer patients in an attempt to salvage the limb and eliminate the need for prostheses. To the end of a revitalizing structural allograft, we have developed a combination gene therapy-tissue engineering approach that introduces angiogenic, osteoclastogenic and osteogenic signals on the cortical surface via immobilized recombinant adeno-associate virus (rAAV) [6,7]. The utility of this rAAV-coating approach has subsequently been realized for soft tissue allografts [8] and stents [9], and the subject has been reviewed [10,11].

Despite the aforementioned problems with massive allograft, and concerns regarding transmission of infectious agents [12,13], cortical allografts have remain the most popular biomaterial used for limb sparing reconstructive surgery due to their biocompatibility and biomechanical properties that have yet to be matched by synthetic biomaterials [14]. However, one major limitation of cortical allograft as a biomaterial is its poor porosity, which compromises its ability to be uniformly and reproducibly coated with therapeutic molecules. In efforts to overcome these shortcomings, investigators have evaluated partially demineralized cortical bone coated with hydrophilic coatings including biodegradable foams of polycaprolactone (PCL), poly(propylene fumarate) (PPF) and poly(d,l-lactic-co glycolic acid) (PLGA) with some success [15-17]. In our studies of rAAV-coated allografts, in which a sorbitol solution of the vector is directly applied to the cortical surface, we have consistently found that gravitational pooling of the vector prior to freeze-drying leads to nonuniform distribution [6]. As a consequence, the *in vivo* gene therapy induced bone formation is asymmetric and in most cases limited to one side of the allograft [7]. Thus, if the results of new bone formation and connectivity with the host are to be improved, we must develop an approach that increases the surface distribution of the rAAV coating by increasing surface absorption, without decrease transduction efficiency. To this end, we investigated the effects of acid surface demineralization of human femur cortical allografts on the release and transduction efficiency of rAAV coatings.

Materials and methods

Preparation of mineralized and surface demineralized cortical bone graft wafers

Human cadaver femurs, previously debrieded and soaked in gentamycin solution, were cut into cross-sectional rings ~5mm in width. Surface demineralization of the cortical bone was performed by the Musculoskeletal Transplant Foundation and achieved by tween/trinton treatment for 30 minutes, soaked in H_2O_2 for 15 minutes, sonicated in purified H_2O for 5 minutes, air dried for 15 minutes, soaked in HCl for 5 minutes, sonicated 3 times in purified H_2O for 15 minutes each, and finally soaked in EtOH for 60 min. Acid penetration depth was determined to be 30-80 microns. Experimental bone wafers were prepared from both mineralized and surface demineralized cortical bone rings by cutting them into 10mm X 5mm

X 3mm samples using a diamond saw as we have previously described [18]. The wafers were sterilized under UV light prior to their use *in vitro* and *in vivo* experiments.

Preparation of rAAV-Luc

The rAAV vector expressing the firefly luciferase gene (rAAV-Luc) was purchased from the Gene Core Facility of the University of North Carolina, Chapel Hill, North Carolina, USA. The vector was prepared using the help virus free transfection method [19], and the titter of purified stock was determined to be 10^{12} particles/ml.

Coating of cortical bone grafts

An aliquot of the rAAV-Luc stock solution containing 10^7,10^9 or 10^{10} particles of was suspended in 20 μl of a 1% sorbitol-PBS solution, and directly pipetted onto the cortical surface of bone grafts. The coated wafers were frozen on dry-ice and placed at −80 °C for 1 hour before lyophilization as we have previously described [6]. Then the rAAV-Luc freeze-dried coated wafers were stored at −80 °C until they were used.

Scanning electron microscopy (SEM) imaging of mineralized and surface demineralized bone wafers

Both coated and uncoated, non-demineralized bone (NDB) wafers and demineralized bone wafers (DBW) were mounted on a stainless steel stage with conductive tape and a drop of conductive graphite adhesive was used to provide a pathway for electrons from the conductive tape to the nonconductive bone grafts. All samples were freeze-dried and sputter coated to apply a thin and completely continuous layer (~10 nm) of gold. A LEO 982 FE-SEM was then used with an accelerating voltage of 10KV to view the surface microarchitecture of the grafts. To analyze the coating of the bone grafts and surface microarchitecture, micrographs were taken 9X and 3000X magnification of the specimens.

Quantification of surface coating on cortical bone wafers

30μl of a 1% sorbitol solution of PBS with red food coloring was pipetted onto NDB or DBW (n=4), and freeze-dried. A digital photograph was taken of the wafers and the % surface coating was quantified using NIH Image by manually tracing and dividing the red surface area by the total wafer surface area.

In-vitro rAAV-Luc transduction assays

C3H10T1/2 cells (10^5 per well in 12-well plates) were grown to confluence in BME supplemented with 2mM L-glutamine, 100units/ml penicillin, 100units/ml streptomycin, and 10%FCS (Hyclone, Logan, UT). To characterize rAAV-Luc transduction with DBW, four groups of C3H10T1/2 monolayers were studied: Group1 untreated cells; Group2 cells incubated with soluble rAAV-Luc only; Group 3 cells incubated with soluble rAAV-Luc in the presence of DBW; and Group 4 cell incubated with rAAV-Luc coated DBW. After 12hr, 24hr, 36hr, 48hr, 60hr, or 72hr of culture at 37 °C, the cells were harvested and assayed for firefly luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Cat. # E1980) and a single-sample luminometer as we have previously described [8].

In-vivo rAAV-Luc transduction assays

The transduction efficiency of rAAV-Luc coated NDB and DBW *in vivo* was determined via longitudinal bioluminescent imaging (BLI) as previously described [20,21]. All animal studies were conducted in accordance with principles and procedures approved by the University of Rochester Committee for Animal Resources. Surgeries were performed using an aseptic technique. 8-week-old female or male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME)

were anesthetized, an 8- to 10-mm-vertical incision was made on the right leg, and quadriceps femoris muscle was exposed. A small pocket was created at the top of the quadriceps femoris muscle by blunt dissection for transplantation of rAAV-Luc coated wafers. The skin was closed with 4-0 silk suture, and the mice were allowed to move freely after recovery from anesthesia. BLI was performed on days 1, 3, 5, 7, 10, 14, 21, and 28 following intraperitoneal injection of D-luciferin potassium salt (Xenogen, Cranbury, NJ) in an IVIS 100 Bioluminescence Imaging System (Xenogen, Cranbury, NJ) as we have described previously [8]. Measurements of the total integrated light signal (photons emitted/cm^2/s) emitted from a standardized region of interest in a standard time interval (5-minute exposure) was computed with the Living Image software (Xenogen, Cranbury, NJ), and the BLI images were generated by overlaying the bioluminescent light intensity map on a reflective-light photograph of the mouse.

Statistical analysis

All values are presented as mean ± SD. Statistical significance was determined using a two-sided *t*-test for two group comparisons, or two-way ANOVA with Bonferroni post-hoc tests for multiple group comparisons, where $p<0.05$ was considered to be significant.

Results

Effects of acid demineralization on cortical allograft bone morphology and surface coating properties

To assess the microscopic effects of surface demineralization on human femoral allograft bone, SEM was performed on NDB and DBW (Figure 1A). The result demonstrated that demineralization markedly increases the porosity of the surface, as evidenced by the large honeycomb space. To determine the effect of demineralization on rAAV-Luc coating efficiency, the vector was applied to NDB and DBW in a 1% sorbitol solution containing red food color and freeze-dried (Figure 1B-C). Quantitation of the surface coating revealed that demineralization caused a significant 21.1 % (73.6 ± 3.9 % vs. 52.5 ± 2.6 %; $p<0.001$) increase in surface area coating versus mineralized controls.

Effects of surface demineralization on rAAV-Luc transduction efficiency in vitro

In order to determine if the demineralization treatments had any effects on the rAAV vector, we performed *in vitro* dose response and time course transduction experiments. For these studies we chose to use the C3H10T1/2 cell line, since it is representative of the mesenchymal progenitor cell targets of the gene therapy and has been demonstrated to mediate efficient bone formation *in vivo* when transfected with osteogenic genes [22,23]. Figure 2A demonstrates the significant increase in gene expression achieved by increasing the multiplicity of infection (MOI) of soluble rAAV-Luc and the incubation period with cultures of C3H10T1/2 cells. However, to directly test if the demineralization had any effects on rAAV-Luc transduction *in vitro* C3H10T1/2 cells were incubated: i) alone, ii) with soluble rAAV-Luc, iii) with uncoated DBW and soluble rAAV-Luc, or iv) with rAAV-Luc coated DBW, and assayed for luciferase activity after 12hr, 24hr, 36hr, 48hr, 60hr, or 72hr (Figure 2B). The data demonstrated that freeze-drying results in a delay in the peak gene expression from 12hr to 60hr, as would be expected based on the rehydration and infection kinetics. However, no significant differences were observed in the peak level of rAAV-Luc gene expression, demonstrating that the demineralization process does not affect the net delivery of the vector.

Effects of surface demineralization on rAAV-Luc transduction efficiency in vivo

Based on the positive *in vitro* findings, we examined the effects of the demineralization process on freeze-dried rAAV-Luc transduction *in vivo* via longitudinal BLI. First we evaluated the dose dependent transduction efficiency following surgical implantation of rAAV-Luc coated

DBW containing 10^7, 10^9 or 10^{10} particles (Figure 3). At the highest dose, significant transgene expression could be detected on day 5, which peaked around day 10, and decreased to baseline levels by day 21. Although less efficient, similar results were obtained with the lower titers consistent with the predicted dose response. We also directly compared the *in vivo* transduction efficiencies of rAAV-Luc coated NDB versus DBW (Figure 4). These results failed to demonstrate any significant differences between the two groups, indicating that rAAV-coated mineralized and demineralized cortical allografts have the same release kinetics and transduction efficiencies *in vivo*.

Discussion

While the osteoconductive and biomechanical properties of cortical allografts make them valuable for limb sparing surgery from segmental defects, their lack of osteoinductive and osteogenic potential has led to poor long-term clinical results [3-5]. Thus, investigators have been working towards developing tissue engineering approaches to overcome this limitation. While addition of bone morphogenetic proteins (BMP) to cancellous allograft bone has proven to be remarkably successful for cavitary bone defects [24], fracture healing [25], and spinal fusion [26,27], the same is not true for large segmental defects that require exogenous BMP activity for at least one week [22]. As a result, stem cell [28-31] and gene therapy [10,32-39] approaches have been proposed as persistent osteogenic adjuvants for massive bone defects. However, a major challenge for this tissue engineering approach is the generation of a biomaterial that has both biomechanical properties required for structural grafting, and absorptive properties required for retention of the therapeutic for *in vivo* delivery. While initial attempts to utilize a biodegradable hydrophilic scaffold that can be wrapped around a cortical allograft have demonstrated some potential [15-17], further work is required for them to achieve clinical utility. Thus, we have proposed a less complicated approach to this problem, which is to render the cortical surface more absorbent via demineralization. In so doing, we have arrived at a clinically-relevant method that allows for uniform coating of soluble osteogenic factors on the surface of the graft. However, future *in vivo* transduction experiments are warranted to confirm the uniform distribution of the rAAV-coating.

A potentially important serendipitous find with these rAAV-coated allografts is the one week delay in peak gene expression. By having the peak in target gene expression one week post-op the gene therapy coincides with the end of the inflammatory phase, and initiation of the reparative phase of bone healing [40]. Moreover, the waning of target gene expression at two weeks may also be advantageous to allow timely commencement of the remodeling phase by three weeks, and abate potential concerns about persistent unregulated gene therapy.

One major limitation of this study is that the quadriceps model, which was chosen based on its facility to demonstrate *in vivo* transduction kinetics, does not simulate the bone defect model in terms of its biomechanical stresses and decreased vascularity. Thus, future studies in the femoral defect model will be useful to substantiate the clinical utility of surface demineralized allografts for segmental defects.

Conclusions

We found that our clinically-relevant surface demineralization procedure significantly increased the porosity and absorbency of human cortical bone allografts to allow for more uniform coating of rAAV vectors. This procedure does so in a manner that does not affect rAAV transduction efficiency or release kinetics from the allograft. Small and large animal efficacy studies with demineralized structural allografts coated with freeze-dried rAAV expressing angiogenic, osteogenic and/or osteoclastogenic genes are warranted to further support the clinical utility of this approach.

Acknowledgements

This work was supported by unrestricted education grants from the Musculoskeletal Transplant Foundation, and National Institutes of Health PHS awards HL51818, DK065988, EY005951, DE17096, AR46545, AR51469 and AR54041.

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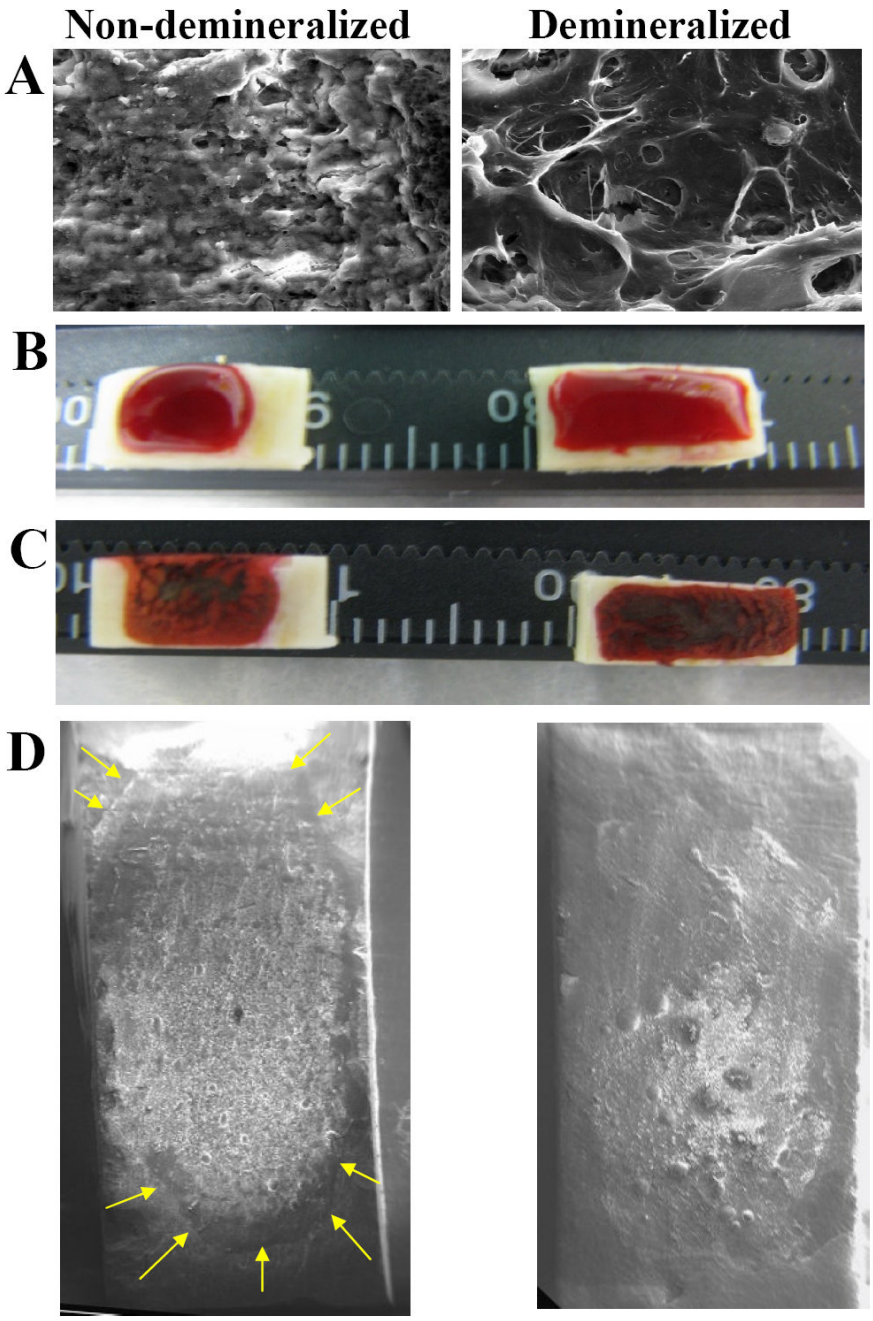

Figure 1. Surface demineralization of cortical allograft bone increases its porosity and absorptive distribution of rAAV-Luc

Scanning electron microscopy (SEM) images of non-demineralized bone (NDB) (left) and demineralized cortical bone wafers (DBW) (right) prior to rAAV coating are shown at 3000 X magnification to demonstrate the marked increase in porosity of the demineralized bone **(A)**. 30μl of a 1% sorbitol solution of PBS with red food coloring containing 10^8 particles of rAAV-Luc was pipetted onto a 10mm NDB (left) or DBW (right), and photographed before **(B)** and after **(C)** freeze-drying. Note that the solution pools in the center of the mineralized bone leading to poor coating after freeze-drying, while the solution is more evenly distributed on the demineralized bone leading to a significant increase in surface coating after freeze-

drying (52.5 ± 2.6 % vs. 73.6 ± 3.9 %; $p<0.001$). Further evidence of improved rAAV-coating uniformity is provided by 9 X SEM images of the freeze-dried wafers **(D)** in which the border of the rAAV coating is clearly visible on the NDB (arrows), while the even distribution on the DBW renders this boarder more transparent.

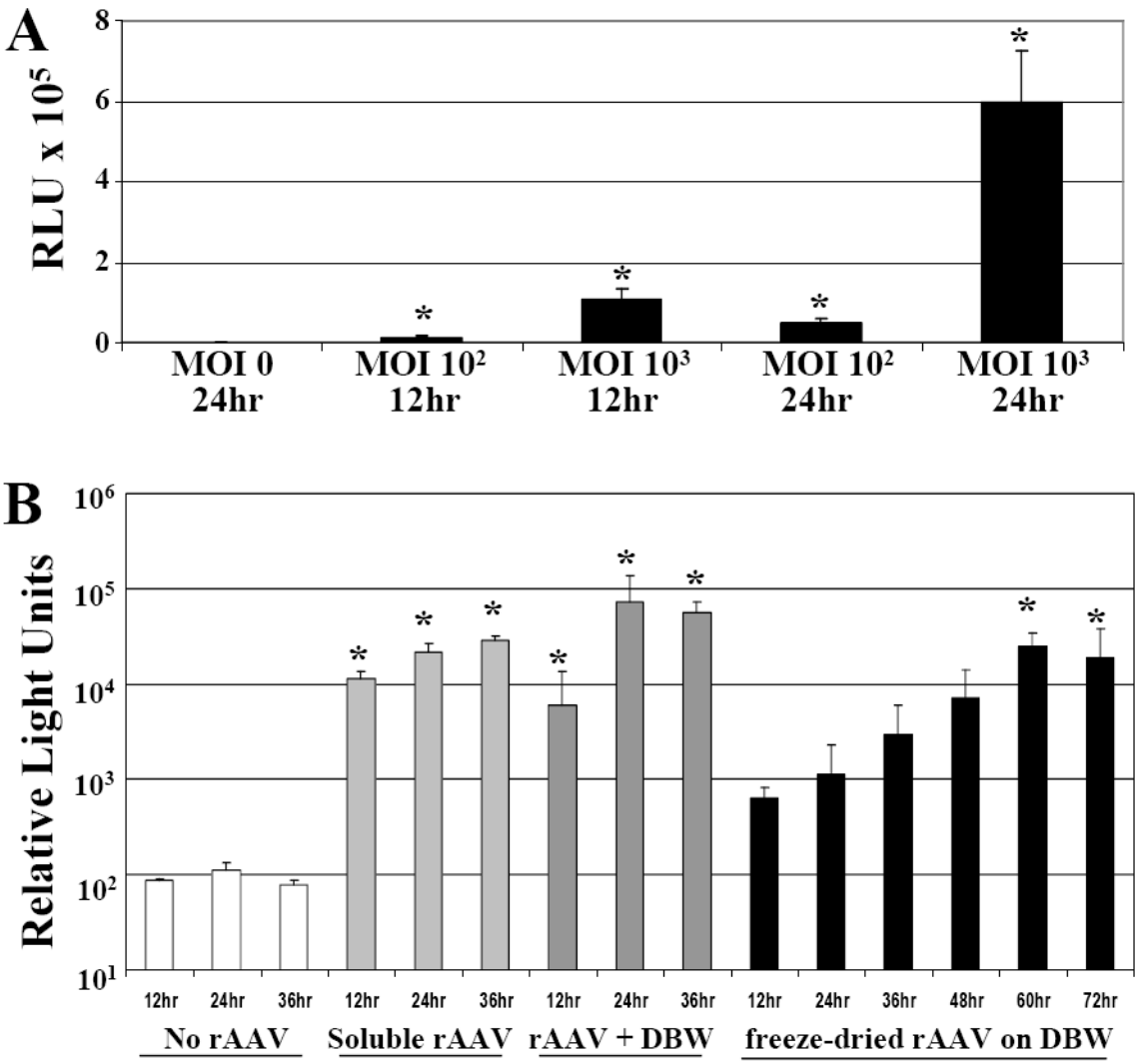

Figure 2. Surface demineralization does not alter rAAV release kinetics and transduction efficiency *in vitro*

The *in vitro* transduction efficiency of the rAAV-Luc vector was determined by infecting a monolayer C3H10T1/2 (10^5 cells per well) at the indicated multiplicity of infection (MOI) and assaying the cells for luciferase activity (relative light units, RLU) at 12hr or 24hr. All transduction conditions lead to a significant increase in RLU versus the uninfected control (asterisk indicates $p<0.05$) **(A)**. The effects of the demineralized bone wafer (DBW) on rAAV transduction and freeze-dried rAAV-Luc release kinetics were determined in C3H10T1/2 transduction experiments *in vitro* **(B)**. Cells were incubated: alone (white bars), with soluble rAAV-Luc (MOI = 100) only (light gray bars), with uncoated DBW and soluble rAAV-Luc (MOI = 100) (dark gray bars), or with rAAV-Luc (MOI = 100) coated-DBW (black bars). RLU assays were performed at the indicated time following treatment. The peak transduction time is indicated by the significant increase over the 12hr control for each group (*; $p<0.05$). No significant effects of the DBW were observed on soluble rAAV-Luc transduction ($p>0.05$). However, the peak of soluble rAAV-Luc transduction occurred at 12hr with or without DBW, while the peak of freeze-dried rAAV transduction occurred at 60hr, but the RLU values of these peaks in the DBW containing cultures were not significantly different ($p>0.05$).

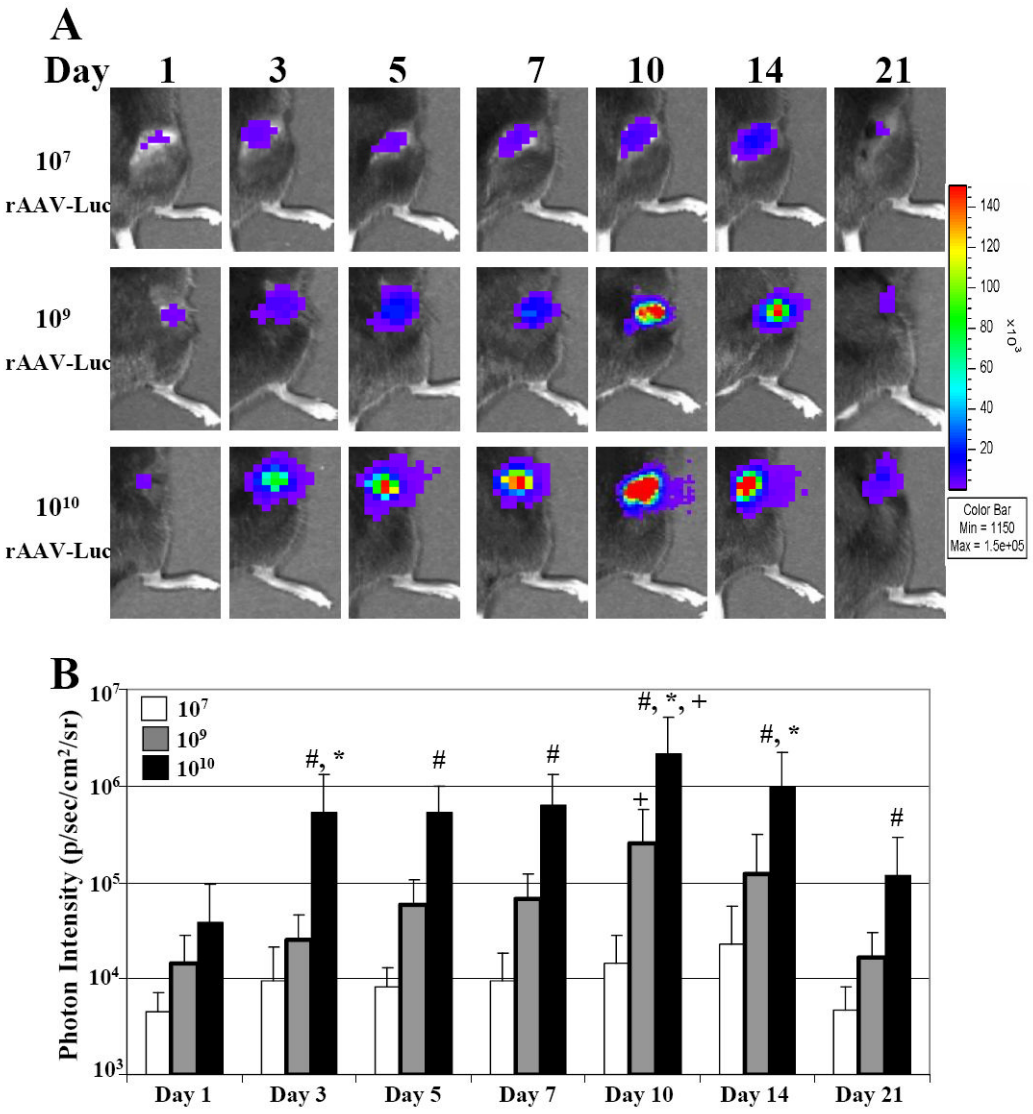

Figure 3. ***In vivo*** **transduction of rAAV-Luc coated demineralized bone wafers**

Demineralized bone wafers (DBW) were coated with 10^7, 10^9, or 10^{10} particles of rAAV-Luc as described in Figure 1, surgically implanted into the right quadriceps of mice (n=6), and temporal bioluminescent imaging (BLI) was performed at days 1, 3, 5, 7, 10, 14, 21. A longitudinal series of BLI photographs of the experimental leg from a representative mouse in each is shown at the indicated time **(A).** The mean BLI ± SD for each dose of rAAV-Luc determined at each time point is presented on a log-scale vertical axis **(B)**. + indicates a significant increase in peak intensity on Day 10 vs. Day 1 for both the 10^9 and 10^{10} groups ($p<0.001$). # indicates significant differences in BLI for the 10^{10} group versus the 10^7 group ($p<0.01$) at the indicated time point. * indicates significant differences in BLI for the 10^{10} group versus the 10^9 group at the indicated time point ($p<0.05$).

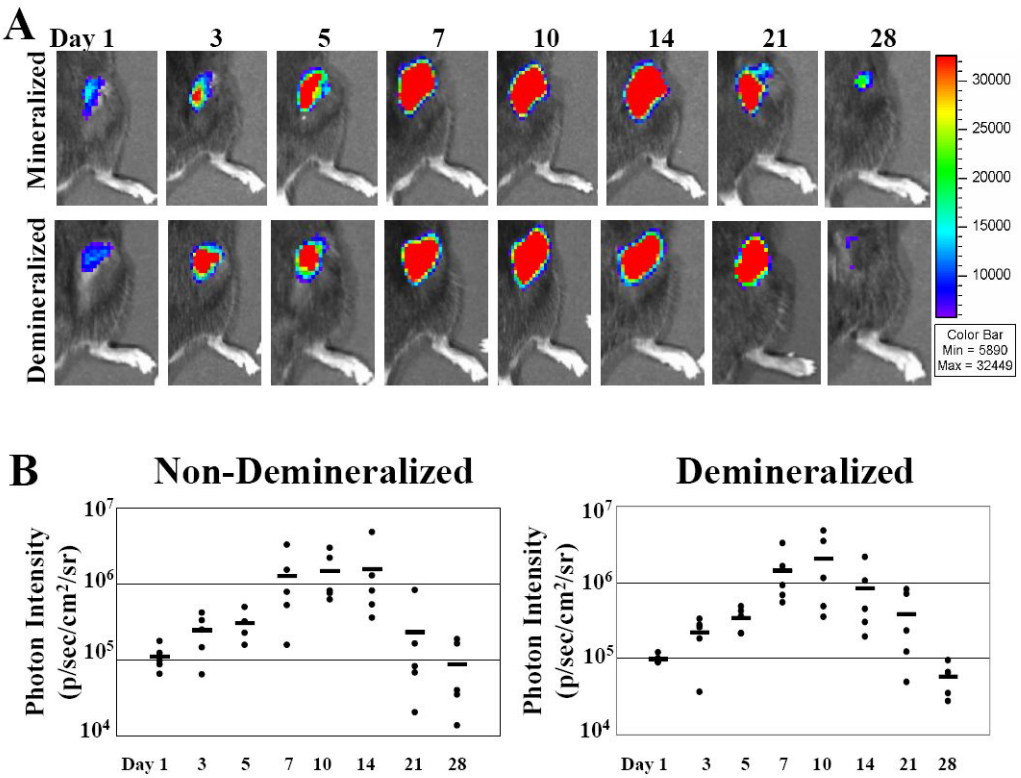

Figure 4. Demineralization of cortical allograft bone does not affect the rAAV-coating release kinetics

Non-demineralized bone (NDB) and demineralized bone wafers (DBW) were coated with 10^{10} rAAV-Luc particles and surgically implanted into mice (n=5) as described in Figure 4. Longitudinal BLI images of a representative mouse from each are shown **(A).** The BLI signal on days 1, 3, 5, 7, 10, 14, 21, and 28 of each mouse is presented **(B).** No significant differences in the BLI signal could be observed between the two groups at any time point ($p>0.05$). Note that the peak BLI for both groups occurs on day 7 with no significant changes between day 7 and 14 for either group ($p>0.05$), but drops to levels below Day 1 by Day 28 for both groups.