

L. Moore¹, M. Gatica¹, H. Kim¹, E. Osawa²,
and D. Ho^{3,4*}

¹Biomedical Engineering, Northwestern University, Evanston, IL, USA;

²NanoCarbon Research Institute, Shinshu University, Ueda, Nagano, Japan;

³Division of Oral Biology and Medicine, Division of Advanced Prosthodontics, The Jane and Jerry Weintraub Center for Reconstructive Biotechnology, UCLA School of Dentistry, Los Angeles, CA, USA; and

⁴Department of Bioengineering, Jonsson Comprehensive Cancer Center, California NanoSystems Institute, UCLA, Los Angeles, CA, USA;

*corresponding author, dean.ho@ucla.edu

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APPENDIX

MATERIALS & METHODS

All cell culture materials and Quanti-iT™ PicoGreen® kits were acquired from Life Technologies (Carlsbad, CA, USA). Recombinant human BMP-2 was obtained from Medtronic Sofamor Danek (Minneapolis, MN, USA). Recombinant human FGF-basic was acquired from EMD Millipore Corporation (Billerica, MA, USA). All components of the cell lysis solution and bovine serum albumin (BSA) were acquired from Sigma Aldrich (St. Louis, MO, USA). Nanodiamonds were acquired from Nanocarbon Research Institute (Nagano, Japan). QUANTI-Blue™ was purchased from InvivoGen (San Diego, CA, USA). All materials related to particle sizing were acquired from Malvern Instruments (Worcestershire, UK). ELISA kits for human BMP-2 and FGF-basic were acquired from R&D Systems (Minneapolis, MN, USA). Cells were purchased from ATCC (Manassas, VA, USA).

Protein Release

Solutions were synthesized at a ratio of 80:1 ND: BMP-2 (by mass). After synthesis, particles were re-suspended in PBS that had been pH-adjusted by the addition of 1 M HCl. Suspensions were incubated for the stated amount of time at 22°C with agitation (600 RPM), after which solutions were pelleted by centrifugation for 15 min at 16,200 x g at 4°C, and supernatants were collected and analyzed for protein content by means of a Micro BCA™ Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Absorbance was measured at 562 and 900 nm and was compared with both a standard curve generated by the kit and ND controls without protein. All experiments were performed in triplicate.

ELISA

The concentrations of BMP-2 and FGF-basic in cell culture supernatants were analyzed in commercially available ELISA kits. The manufacturer's protocols were used unless otherwise specified. For the BMP-2 ELISA, supernatants were diluted either 1:500 or 1:1,000 in PBS, and the standard curve was

Multi-protein Delivery by Nanodiamonds Promotes Bone Formation

Appendix Table. FTIR Peaks (cm⁻¹)

ND Only	ND-BF	ND-BMP	ND-BSA
3,430	3,425.61	3,428.3	3,437.13
2,922.92	2,922.3	2,924.59	2,923.6
1,635.25	1,657.47	1,657.49	1,652.94
-	1,649.31	1,649.2	1,646.72
-	1,564.07	1,564.31	1,635.69
-	1,546.78	1,547.11	1,558.81
-	1,536.19	1,535.35	1,540.42
1,384.02	1,384.18	1,384	-
1,260.56	-	-	-
-	1,205.5	1,204.51	-
-	1,138.31	1,135.54	-
1,054.58	1,049.07	1,048.139	1,054.889
803.48	802.255	802.51	802.255

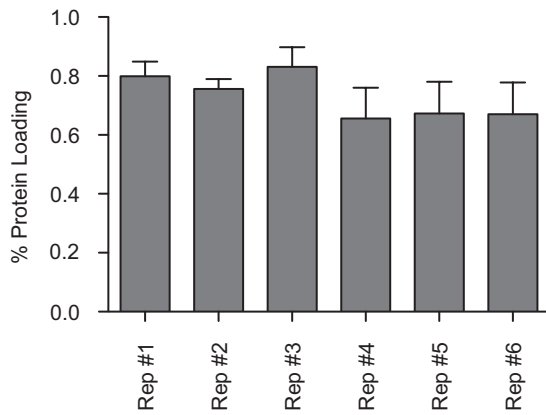
generated from the recombinant protein. For the bFGF ELISA kit, the supernatants were analyzed without dilution, and a standard curve was generated from the recombinant protein at 0.1 to 6.4 ng/mL. All ELISA results represent the mean and standard deviation from a minimum of 2 independent experiments.

Sizing

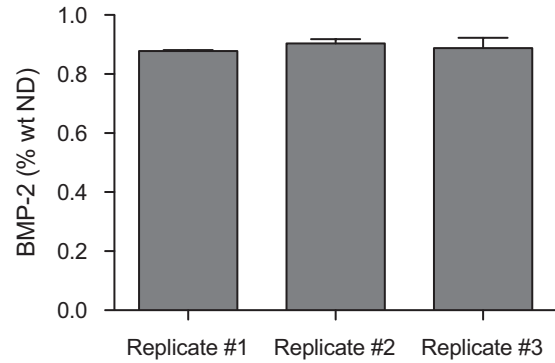
Solutions were synthesized with ratios of ND:BMP-2 of 1:1 to 160:1 (by mass). Final solution concentration was 66.6 µg/mL ND. Average particle diameter was acquired on a Zetasizer ZSP (Malvern Instruments, Worcestershire, UK) with low-volume cuvettes. Measurements shown are the z-average diameter acquired from 3 readings on each sample. Mean and standard deviation displayed are derived from readings on 3 batches.

Cell Culture

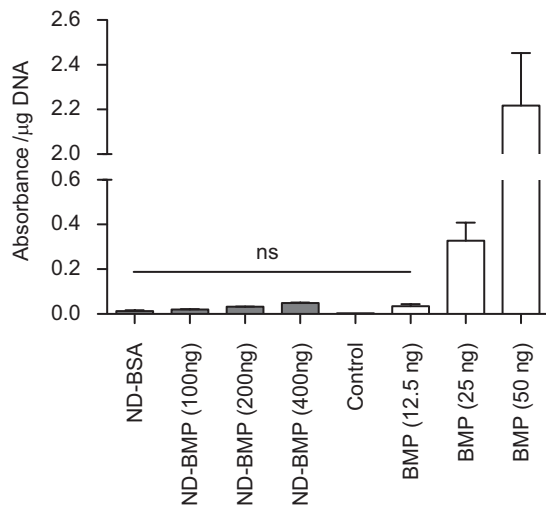
C2C12 mouse myoblast cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C and 5% CO₂. Cells were passaged a maximum of 8 times and were never allowed to reach 100% confluence.



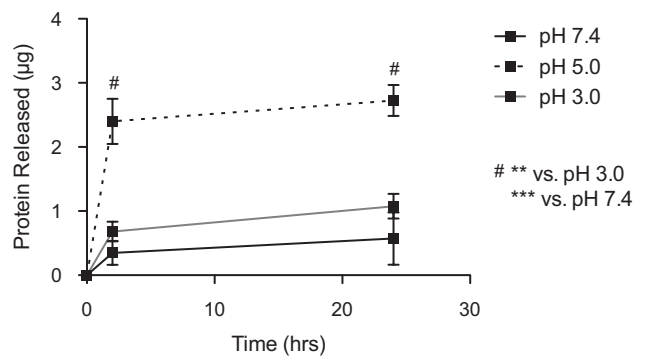
Appendix Figure 1. Assessment of total protein loading onto NDs by BCA assay. Assay demonstrated consistent loading of protein into ND clusters across replicates and days, with an average loading of 73% of the original protein, equivalent to 45.6% wt ND.



Appendix Figure 2. Assessment of BMP-2 loading into ND clusters by C2C12 cell alkaline phosphatase production. C2C12 cells were incubated with the supernatants from 3 separate batches of ND-BMP for 72 hrs and then assayed for alkaline phosphatase production, normalized to DNA content. Samples were compared with a standard curve generated by the incubation of cells with known concentrations of BMP-2 to determine the amount of BMP-2 loaded into ND clusters.



Appendix Figure 3. Delayed release of BMP-2 from ND clusters. C2C12 cells were incubated with ND-BMP or BMP-2 for 24 hrs prior to being washed and assayed 48 hrs later. Cellular response to ND-BMP was reduced compared with that after 72-hour incubation, demonstrating delayed release of BMP-2 from ND clusters. Doses listed indicate initial synthesis conditions.



Appendix Figure 4. Total protein release from ND-BMP clusters was dependent on solution pH (** $p < .01$, *** $p < .001$).