

Supporting Information

Tough and Immunosuppressive Titanium-Infiltrated Exoskeleton Matrices for Long-Term Endoskeleton Repair

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Supplementary Materials and Methods

Fabrication of Chitin Nanofibrous Membrane (ChNM)

Chitin powder (20 g) was refluxed in 500 ml of 20% NaOH aqueous solution at 150 °C for 6 h for partial deacetylation. The partially deacetylated chitin was then washed with deionized (DI) water thoroughly and allowed to stand at room temperature for 1 h in DI water. For effective nanofibrillation of chitin, positive charges were introduced to the amine groups on the surface of deacetylated chitin by adding acetic acid at pH 3. The cationized chitin was loaded into a high-speed grinder (MKCA6-2, Masuko Sangyo co., ltd., Japan) for mechanical disintegration at a speed of 1500 rpm with a clearance gauge of -1.5 from zero position; the acidity was maintained at pH 3. The fabricated chitin nanofiber (1%, w/v) fully dispersed in DI water was filtered by passing through the hydrophilic PTFE filter paper under vacuum. Then, the filtered slurry was dried in an oven at 45 °C for 1 h to obtain the chitin nanofibrous membrane (ChNM).

X-ray Diffraction (XRD) Analysis

X-ray diffraction (XRD) experiment was conducted with a D/MAX-2500/PC (Rigaku, Japan) under 40 kV and 100 mA Ni-filtered CuK_α radiation ($\lambda = 1.5418 \text{ \AA}$). The XRD patterns were recorded in an angular (2θ) range of 5° to 45° with a step size of 0.02° and a scanning speed of 1° min^{-1} . After accumulating pulses at each step, the full-width at half-maximum (FWHM) was determined by the Lorentzian peak function using ORIGIN Pro 9.0 to estimate the nanocrystallite size in a direction perpendicular to the plane (from Scherrer equation). The crystallinity index (CrI) was calculated by $\text{CrI} = \{\text{intensity of the (110) plane} - \text{intensity of the amorphous scattering at } 16^\circ\} \times 100 / \text{intensity of the (110) plane}$.¹

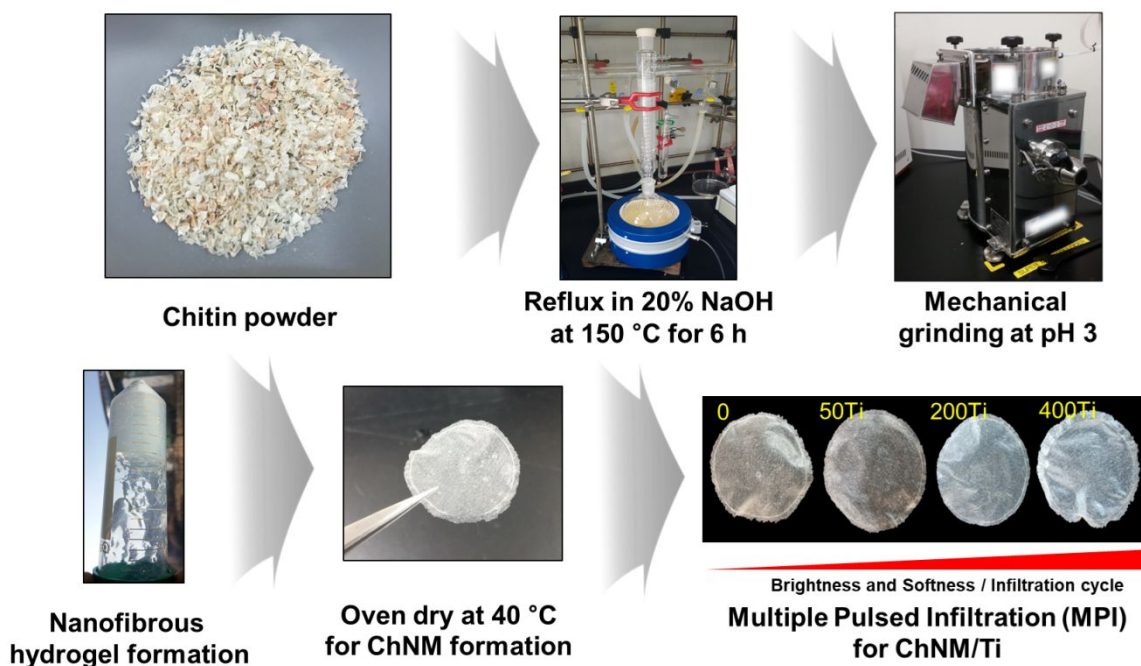


Figure S1. Fabrication process of chitin nanofibrous membrane/Ti hybrid (ChNM/Ti). Raw chitin powder from crab shell was partially deacetylated by reflux process. Then cationized (amine functionalized) chitin was simply grinded to make chitin nanofiber dispersed in water. Then, oven dried membranes were applied to different cycles of TiO₂ ALD (0, 50Ti, 200Ti, and 400Ti).

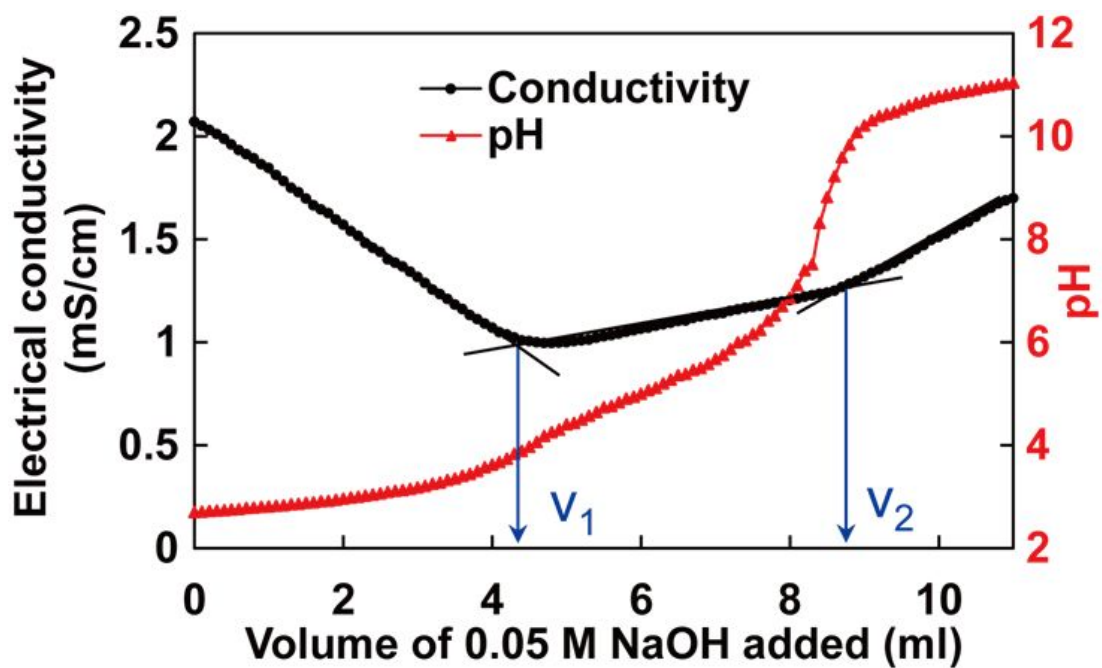


Figure S2. Conductometric titration to measure degree of deacetylation (DD) after nanofibrillation.¹ From the slope change during the addition of 0.05 M NaOH, amount of amine groups on chitin was calculated. This indicates that partially deacetylated chitin nanofiber was successfully fabricated (DD ~37%).

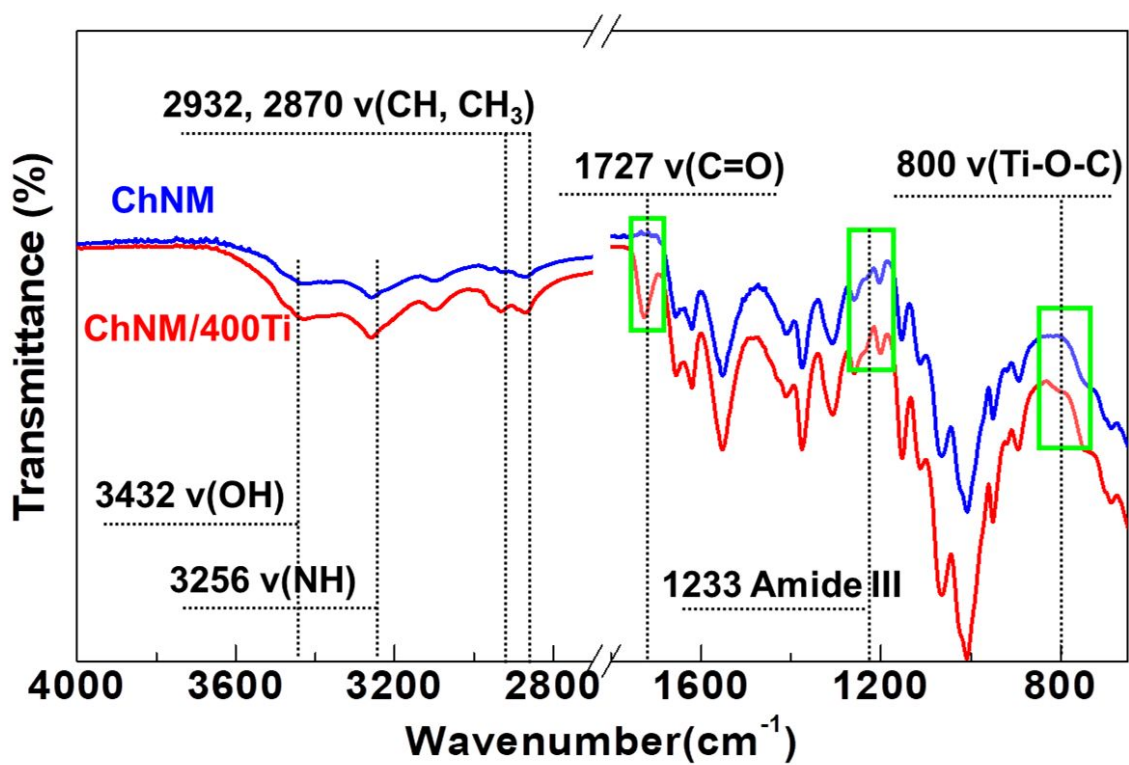


Figure S3. FT-IR spectra of ChNM and ChNM/400Ti. The green boxes indicate significantly changed characteristic peaks after 400 ALD cycles.

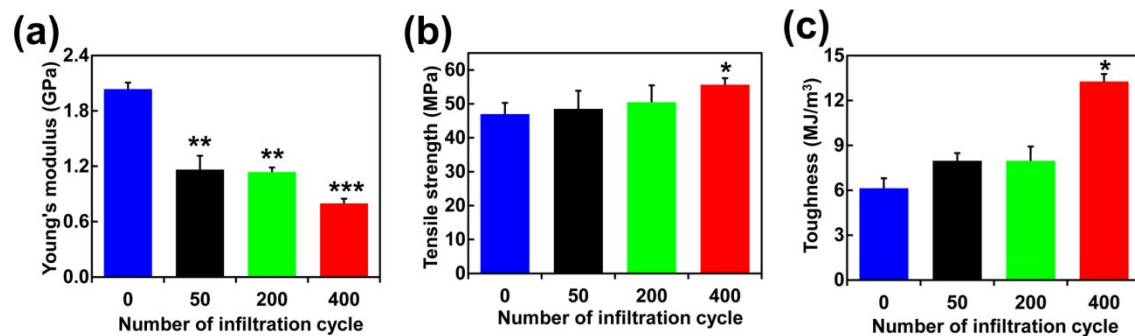


Figure S4. Statistical evaluation of mechanical properties. (a) Young's modulus, (b) tensile strength, and (c) toughness. All experiments were performed several times ($n=9$; the mean value \pm SD) and significant differences compared to pristine were confirmed statistically ($*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

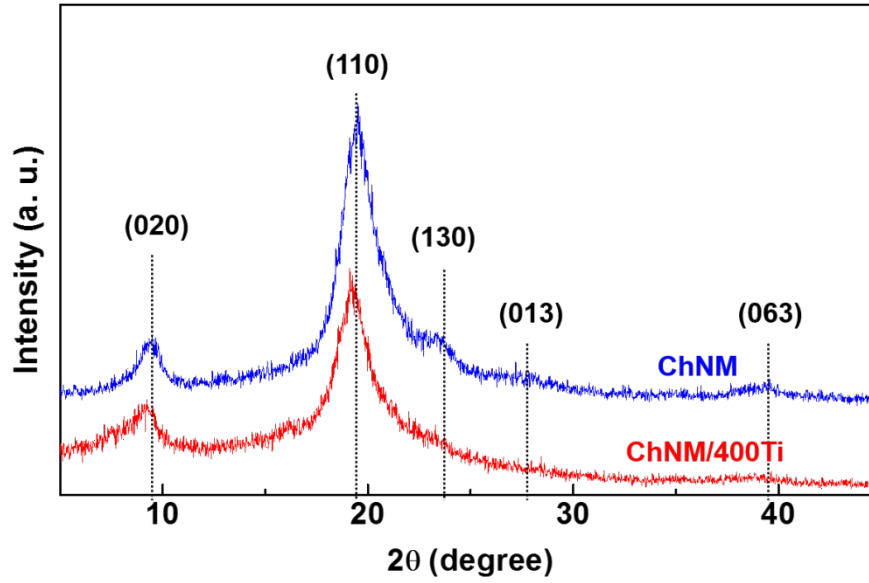


Figure S5. X-ray diffraction patterns of ChNM and ChNM/400Ti. Characteristic peaks of chitin appear at 9.4° , 19.6° , 22.8° , 25.8° , and 39° corresponding to (020), (110), (130), (013), and (063) planes.^{2,3} After Ti infiltration, (130), (013), and (063) peaks disappeared, which suggests serious distortion of the chitin crystal structure. In particular, the redshift of (110) peak suggests an increase in the lattice spacing of the chitin crystallite by Ti infiltration.

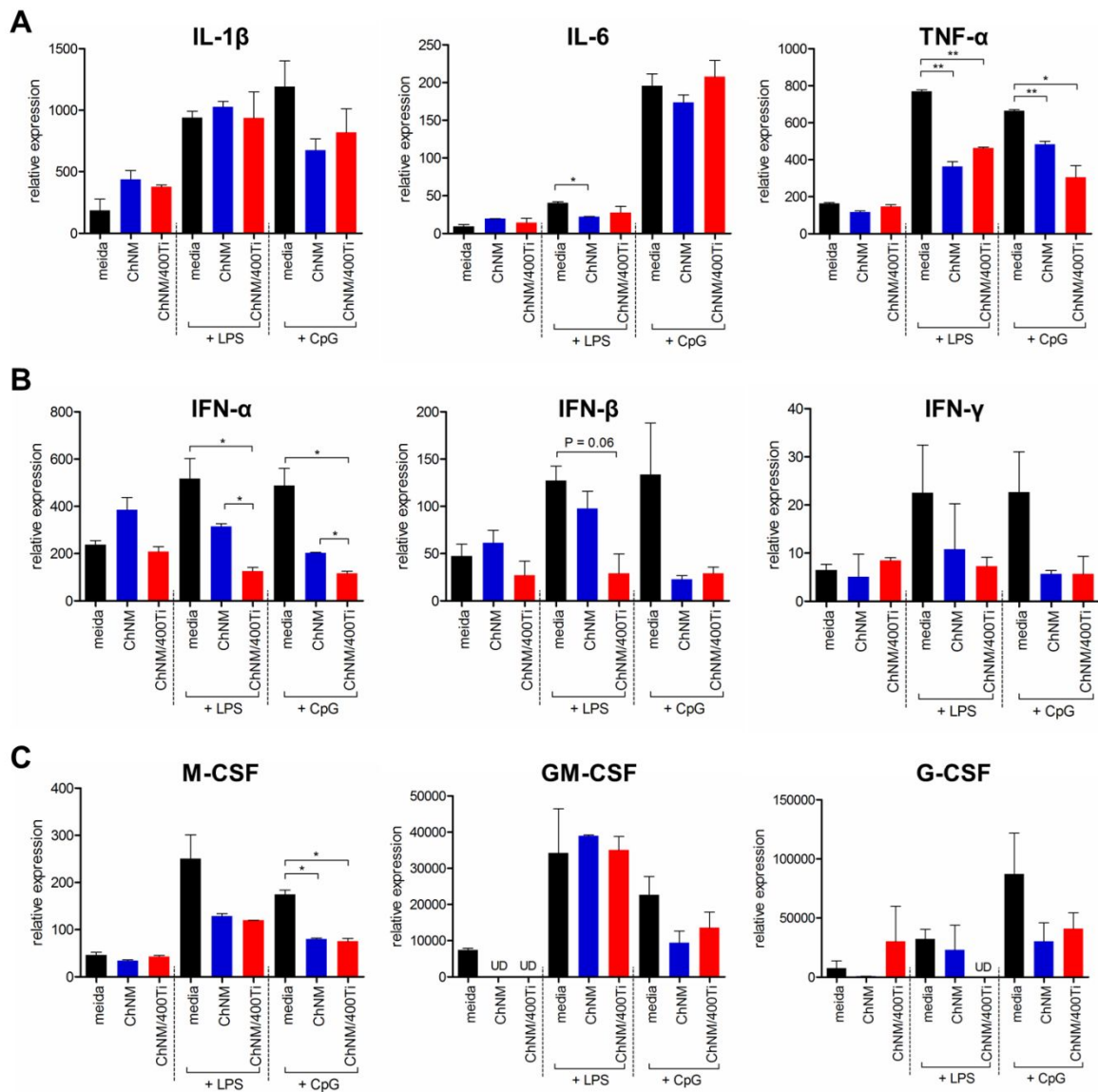


Figure S6. Real-time PCR data indicating expression levels of cytokines from MACS-sorted dendritic cells (MACS-DCs). (a) Pro-inflammatory (IL-1 β , IL-6, and TNF- α), (b) interferon (IFN- α , β , and γ), and (c) colony-stimulating factor family (M-CSF, GM-CSF, and G-CSF) cytokines. Data are mean \pm SD. All experiments were performed several times (n=3) and significant differences were confirmed statistically (* p < 0.05, ** p < 0.01, and undetected (UD)).

Table S1. XRD peak locations and distances between planes in ChNM and ChNM/400Ti (2 θ : diffraction angle of each peak, d: spacing).

Plane	Location (2 θ) [°]	Spacing d (=1.54/2sin θ) [Å]	Nanocrystallite size ^{c)} [nm]
(020)	9.4	9.42 ± 0.002 ^{a)} 9.79 ± 0.004 ^{b)}	6.20 ± 0.132 ^{a)} 4.80 ± 0.017 ^{b)}
(110)	19.6	4.53 ± 0.002 4.60 ± 0.000	3.98 ± 0.118 4.40 ± 0.023
(130)	22.8	-	-
(013)	25.8	-	-
(063)	39	-	-

^{a)}ChNM; ^{b)}ChNM/400Ti; ^{c)}From Scherrer equation

Table S2. qPCR primer sequences. The following primer pairs were used for qPCR experiments in this study.

Gene ^{a)}	Forward (5' → 3')	Reverse (3' → 5')
L32	GAA ACT GGC GGA AAC CCA	GGA TCT GGC CCT TGA ACC TT
TNF- α	AGG GTC TGG GCC ATA GAA CT	CCA CCA CGC TCT TCT GTC TAC
IL-1 β	AGG TCA AAG GTT TGG AAG CA	TGA AGC AGC TAT GGC AAC TG
IL-6	AAG TGC ATC ATC GTT GTT CAT ACA	GAG GAT ACC ACT CCC AAC AGA CC
IFN- α	TAT GTC CTC ACA GCC AGC AG	TTC TGC AAT GAC CTC CAT CA
IFN- β	CCC AGT GCT GGA GAA ATT GT	CCC TAT GGA GAT GAC GGA GA
IFN- γ	GAG CTC ATT GAA TGC TTG GC	GCG TCA TTG AAT CAC ACC TG
M-CSF	GGT AGT GGT GGA TGT TCC CA	CCA GGA TGA GGA CAG ACA GG
GM-CSF	CCG TAG ACC CTG CTC GAA TA	CCA TCA AAG AAG CCC TGA AC
G-CSF	TGA CAC AGC TTG TAG GTG GC	TCC TGC TTA AGT CCC TGG AG

a) C57BL/6 (B6) mouse gene

References

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- (2) Ifuku, S.; Nogi, M.; Abe, K.; Yoshioka, M.; Morimoto, M.; Saimoto, H.; Yano, H. Preparation of Chitin Nanofiber with a Uniform Width as α -Chitin from Crab Shells. *Biomacromolecules* **2009**, *10*, 1584-1588.
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