Supplementary Data

Supplementary Materials and Methods

Reagents for cell culture

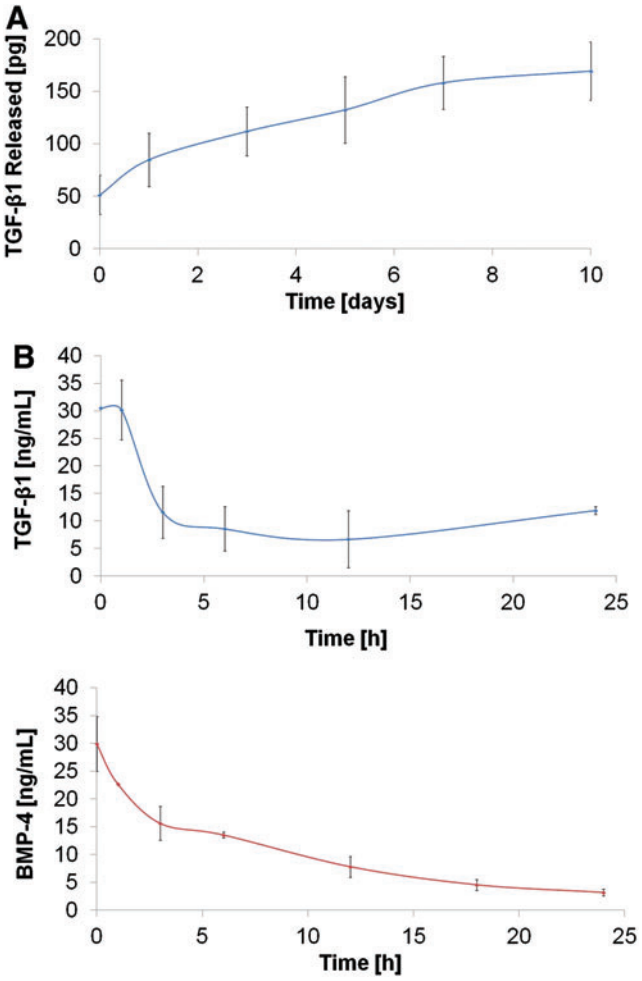
All growth factors and neutralizing antibodies—recombinant human transforming growth factor (TGF)-β3, recombinant human bone morphogenetic protein (BMP)-4, monoclonal mouse TGF-β1/2/3 antibody (anti-TGF-β), monoclonal mouse BMP-4 antibody (anti-BMP-4), and recombinant human TGF-β1—were purchased from R&D Systems (Minneapolis, MN). In addition, the following biochemical supplements were used in cell culture: fetal bovine serum (Gibco, Invitrogen, Carlsbad, CA); penicillin (100 U/mL)–streptomycin (100 μg/mL) (Gibco); dexamethasone (G Biosciences, Maryland Heights, MO); L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO); insulin, transferrin, and selenium (BioWhittaker, Lonza, Walkersville, MD); and β-glycerophosphate (Sigma-Aldrich).

Synthesis of poly(lactide-co-glycolide) microspheres

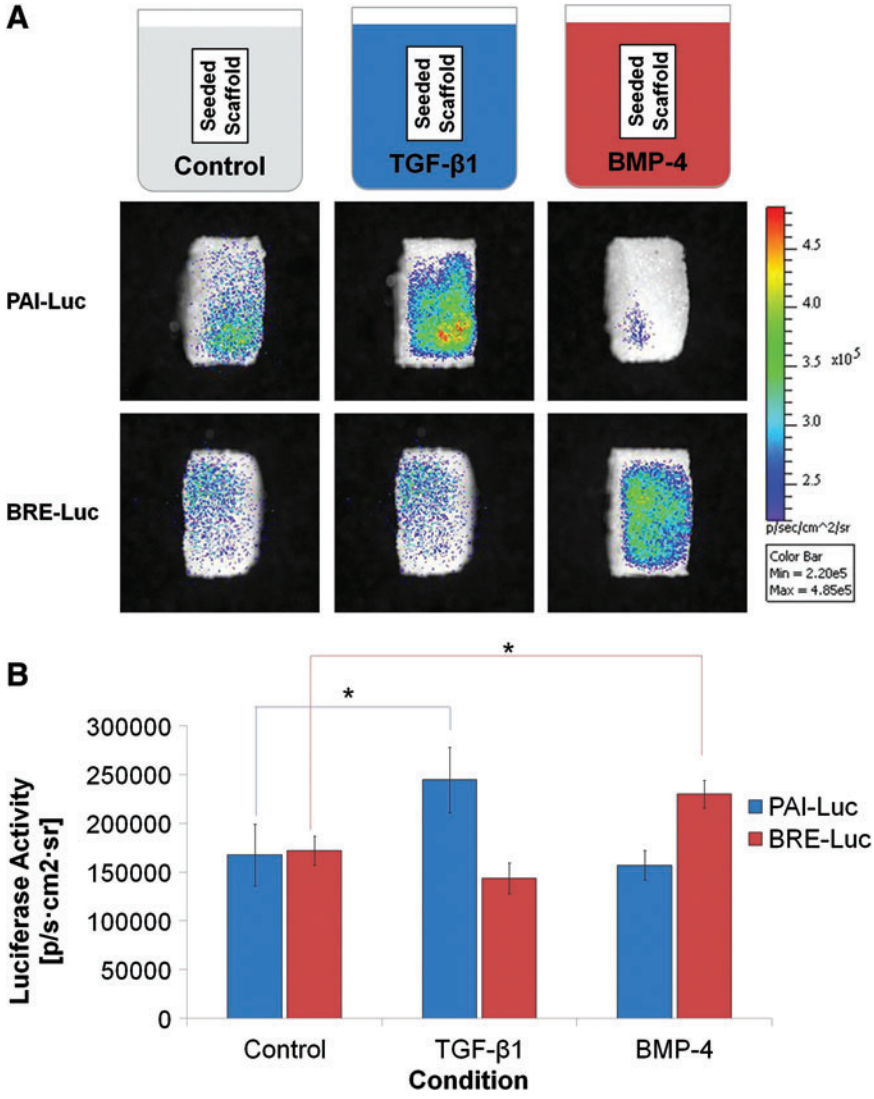
Poly(lactide-co-glycolide) (PLG) microspheres were prepared using a double emulsion technique as described previously[1] to encapsulate TGF-β3 (0.4 ng per mg of microspheres), BMP-4 (0.5 ng per mg of microspheres), insulin (1 μg per mg of microspheres)–transferrin (1 μg per mg of microspheres)–selenium (1 ng per mg of microspheres), BMP-4 (0.5 ng per mg of microspheres), β-glycerophosphate (0.2 mg per mg of microspheres), anti-TGF-β (12 ng per mg of microspheres), or anti-BMP-4 (6 ng per mg of microspheres). Briefly, 100 μL of aqueous microsphere contents (i.e., the proteins and/or small molecules to be encapsulated within PLG microspheres) was pipetted into 1 mL of 5% PLG (8515DLG7E) (Lakeshore Biomaterials, Birmingham, AL) in ethyl acetate (Sigma-Aldrich), and the mixture was sonicated at an amplitude of 40 (VCX 130; Sonics, Newtown, CT) for 15 s. Then, 1 mL of 1% polyvinyl acetate (PVA; Sigma-Aldrich) in 7% ethyl acetate was added, and the mixture was vortexed at maximum speed for 15 s. The mixture was then transferred into a bath of 0.3% PVA in 7% ethyl acetate and stirred continuously at 500 rpm under a chemical hood for 3 h to promote precipitation of the microspheres by solvent evaporation. The microspheres were then filtered through a 0.2-μm-diameter filter (Nalgene, Rochester, NY), collected by centrifugation at 2200 rpm for 10 min (Eppendorf, Hauppauge, NY), lyophilized for 72 h (Labconco, Kansas City, MO), and stored at −20°C.

Synthesis of rhodamine-conjugated silica nanoparticles

A solution of 1% rhodamine isothiocyanate (RITC; Sigma-Aldrich), prepared by dissolving 10 mg RITC, was dissolved in 1 mL of ethanol with 44 μL of (3-aminopropyl) trimethoxysilane (Sigma-Aldrich), and a solution 5% tetraethyl orthosilicate (TEOS; Sigma-Aldrich) was prepared by mixing 0.5 mL TEOS with 7.4 mL of anhydrous ethanol, 1 mL of molecular-grade water, and 0.1 mL of ammonium hydroxide (NH_4OH; Sigma-Aldrich). To conjugate rhodamine to the silica, 100 μL of the RITC solution was added into the TEOS solution, and the mixture was rapidly stirred for 24 h. The fluorescent nanoparticles were then collected by centrifugation at 10,000 rpm for 5 min (Eppendorf, Hauppauge, NY) and resuspended in 2 mL of molecular-grade H_2O.



SUPPLEMENTARY FIG. S1. Growth factor release and degradation kinetics. **(A)** Cumulative release curve of transforming growth factor (TGF)-β1 from single-layer scaffolds. Scaffolds containing TGF-β1 were incubated in phosphate-buffered saline (PBS) under culture conditions; PBS was collected and replaced after 1, 3, 5, 7, and 10 days; and growth factor concentration in collected PBS was measured by enzyme-linked immunosorbent assay (ELISA) ($n=4$). The TGF-β1 release profile was fitted to a third-order time-dependent polynomial to acquire the protein release function used in mathematical modeling. **(B)** Degradation/uptake profiles of TGF-β1 (*upper*) and bone morphogenetic protein (BMP)-4 (*lower*) in the presence of mesenchymal stem cells (MSCs). MSCs were cultured in media containing growth factors, and growth factor concentrations were measured in media by ELISA ($n=2$). Degradation profiles were fitted to exponential decay equations to acquire the half-life of each species.



SUPPLEMENTARY FIG. S2. Specificity of luciferase reporter cells to TGF-β1 and BMP-4. Single-layer poly(lactide-co-glycolide) scaffolds uniformly seeded with PAI-Luc or BRE-Luc cells were cultured in complete Dulbecco's Modified Essential Media (DMEM) supplemented with no growth factors (*left*), 10 ng/mL TGF-β1 (*center*), or 30 ng/mL BMP-4 (*right*). Luciferase activity was recorded after 24 h. **(A)** Representative images taken with the Xenogen IVIS-200 system. **(B)** Quantification of photon flux density ($n = 3$). *Asterisks* indicate statistical significance at $p < 0.05$.

Three-dimensional printing

Customized scaffold molds were designed in SolidWorks (Dassault Systems, Waltham, MA) using a digital model of an adult human femoral condyle that had been reconstructed from computed tomography imaging (3D ContentCentral; Dassault Systems). Molds were fabricated at the 1:8 anatomical scale in VeroBlue polyjet resin using a Connex500 3D printer (Objet, Billerica, MA).

Equation 1. Function for protein release from PLG scaffold

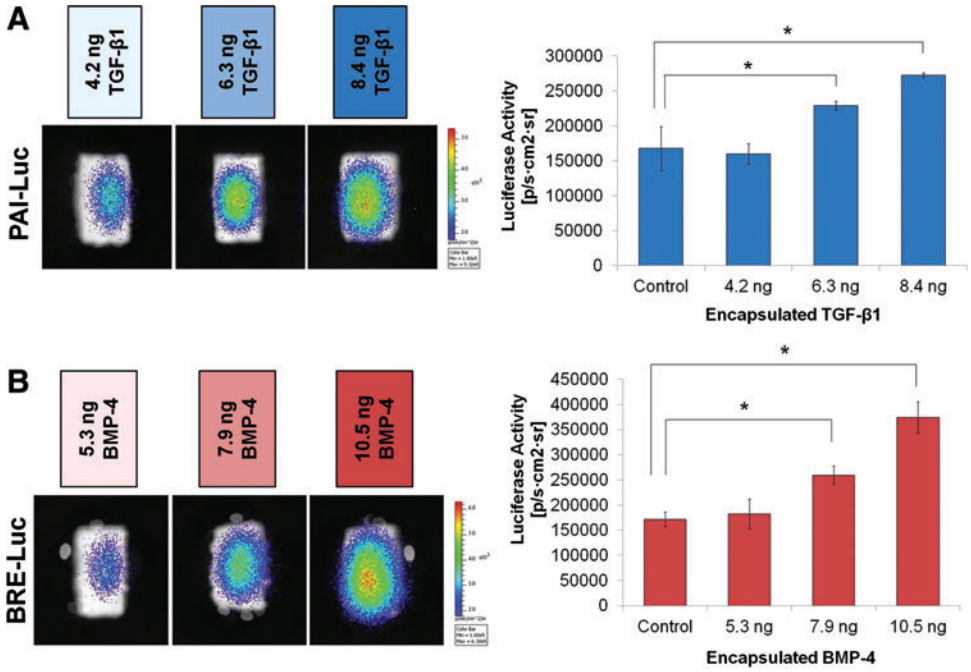
$$C = C_{tot}[(5.748 \times 10^{-19})t^3 - (1.503 \times 10^{-12})t^2 + (1.633 \times 10^{-6})t + 0.0299)]$$

Calculated from a third-order polynomial fit for TGF-β1 release from a single-layer PLG scaffold, with $\boldsymbol{R^2 = 0.9891}$.

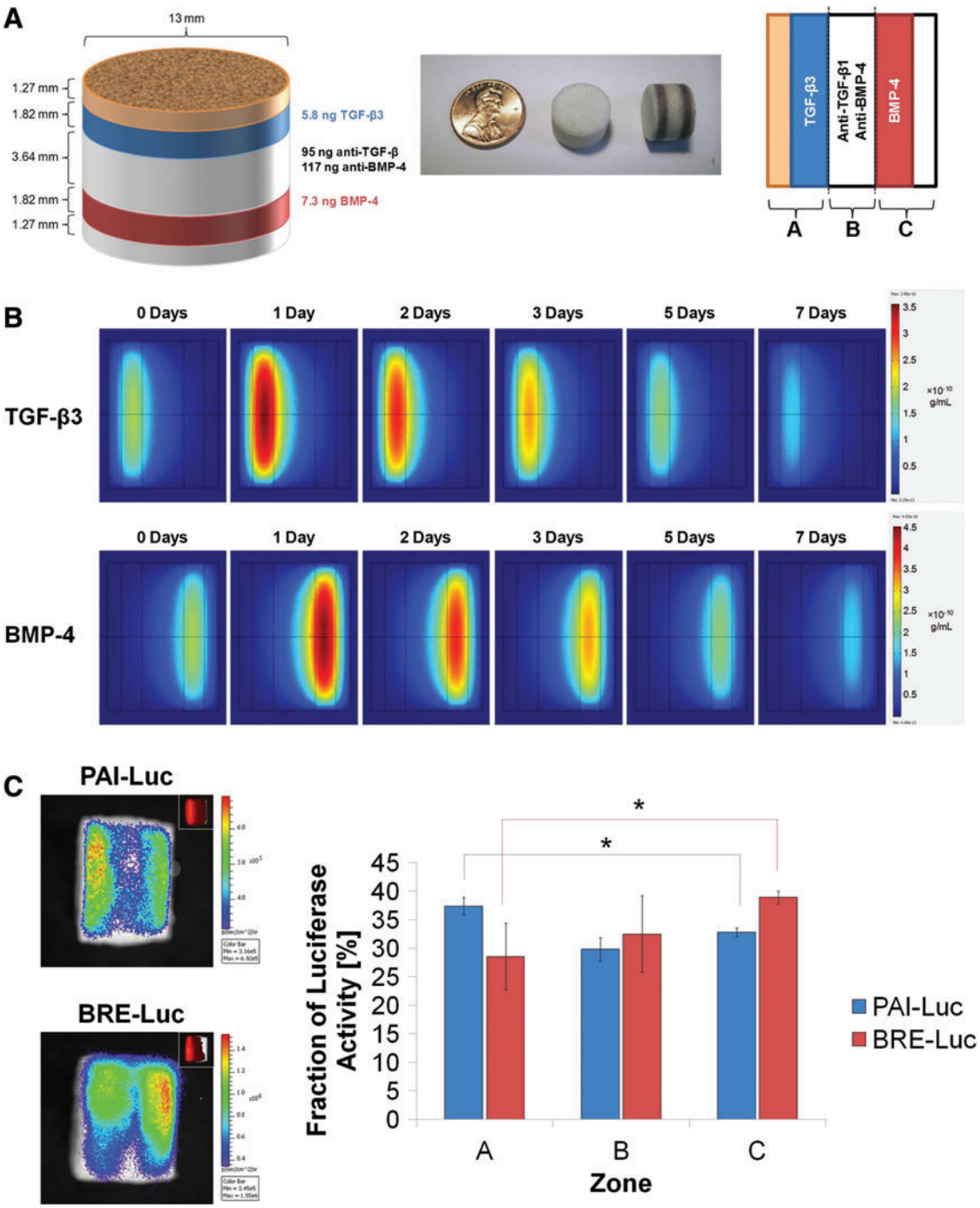
$\boldsymbol{C}$ denotes the cumulative protein released from the scaffold layer in units of g/mL, $\boldsymbol{C_{tot}}$ represents the total concentration of protein in the scaffold layer after salt leaching in units of g/mL, and $\boldsymbol{t}$ represents the time after cell seeding in units of s.

Supplementary References

1. Cohen, S., Yoshioka, T., Lucarelli, M., Hwang, L.H., and Langer, R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. *Pharm Res* **8,** 713, 1991.
2. Young, M.E., Carroad, P.A., and Bell, R.L. Estimation of diffusion coefficients of proteins. *Biotechnol Bioeng* **22,** 947. 1980.
3. Morell, A., Terry, W.D., and Waldman, T.A. Metabolic properties of IgG subclasses in man. *J Clin Invest* **49,** 673, 1970.
4. Yuen, W.W., Du, N.R., Chan, C.H., Silva, E.A., and Mooney, D.J. Mimicking nature by codelivery of stimulant and inhibitor to create temporally stable and spatially restricted angiogenic zones. *Proc Natl Acad Sci U S A* **107,** 17933, 2010.



SUPPLEMENTARY FIG. S3. Dose responsiveness of luciferase reporter cells to TGF-β1 and BMP-4. Single-layer scaffolds containing increasing quantities of encapsulated TGF-β1 **(A)** or BMP-4 **(B)** were uniformly seeded with PAI-Luc or BRE-Luc cells. Luciferase activity was recorded after 24 h. Representative images taken with the Xenogen IVIS-200 system (*left*) and quantification of photon flux density (*right*) are shown ($n = 4$). *Asterisks* indicate statistical significance at $p < 0.05$.



SUPPLEMENTARY FIG. S4. Five-layer scaffold with neutralizing antibodies in the middle layer (second-generation design). **(A)** Schematic of scaffold dimensions and contents after optimization by mathematical modeling (*left*); photographs of five-layer scaffolds, in which selected layers have been labeled with orcein (*middle*); and orientation of scaffolds for mathematical modeling and luciferase imaging (*right*). **(B)** Slice plots illustrating concentration profiles of TGF-β3 (*upper row*) and BMP-4 (*lower row*) over 7 days, as simulated in COMSOL Multiphysics. Values reported in g/mL. **(C)** Representative images (*left*) and quantification (*right*) of photon flux density through scaffold zones at 24 h after seeding with PAI-Luc or BRE-Luc cells ($n = 3$ for each reporter line). *Asterisks* indicate statistical significance at $p < 0.05$.

Supplementary Table S1. Antibodies Used in Western Blotting

Target	*Manufacturer*	*Dilution factor*	*Molecular weight (kDa)*
SRY box 9 (Sox9)	Abcam (Cambridge, MA)	1:500	65
Runt-related transcription factor 2 (Runx2)	Invitrogen (Carlsbad, CA)	1:500	57
Aggrecan (AGG)	Abcam (Cambridge, MA)	1:100	50
Type II collagen (Col-II)	Abcam (Cambridge, MA)	1:500	100
Bone sialoprotein II (BSP-II)	Cell Signaling (Danvers, MA)	1:1000	82
Osteopontin (OPN)	Developmental Studies Hybridoma Bank (Iowa City, IA)	1:500	60
Type X collagen (Col-X)	Abcam (Cambridge, MA)	1:500	66
β-Actin	Cell Signaling (Danvers, MA)	1:10,000	45

SUPPLEMENTARY TABLE S2. PARAMETERS USED IN MATHEMATICAL MODELING

Parameter	*Value*	*Source*
TGF-β3 molecular weight	25.4 kDa	R&D Systems (Minneapolis, MN)
TGF-β3 diffusion coefficient in H_2O	1.2810×10^{-6} cm^2/s	Young–Carroad–Bell method[2]
TGF-β3 diffusion coefficient in PLG scaffold	1.1529×10^{-6} cm^2/s	90% of H_2O diffusivity
BMP-4 molecular weight	26 kDa	R&D Systems (Minneapolis, MN)
BMP-4 diffusion coefficient in H_2O	1.2647×10^{-6} cm^2/s	Young–Carroad–Bell method[2]
BMP-4 diffusion coefficient in PLG scaffold	1.1382×10^{-6} cm^2/s	90% of H_2O diffusivity
Molecular weight of anti-TGF-β	146 kDa	Typical mass of IgG
Anti-TGF-β diffusion coefficient in H_2O	7.1154×10^{-7} cm^2/s	Young–Carroad–Bell method[2]
Anti-TGF-β diffusion coefficient in PLG scaffold	6.4039×10^{-7} cm^2/s	90% of H_2O diffusivity
Molecular weight of anti-BMP-4	146 kDa	Typical mass of IgG
Anti-BMP-4 diffusion coefficient in H_2O	7.1154×10^{-7} cm^2/s	Young–Carroad–Bell method[2]
Anti-BMP-4 diffusion coefficient in PLG scaffold	6.4039×10^{-7} cm^2/s	90% of H_2O diffusivity
Molecular weight of TGF-β3-antibody complex	171.4 kDa	Molecular weight of TGF-β3 + IgG
TGF-β3-antibody complex diffusion coefficient in H_2O	6.7502×10^{-7} cm^2/s	Young–Carroad–Bell method[2]
TGF-β3-antibody complex diffusion coefficient in PLG scaffold	6.0752×10^{-7} cm^2/s	90% of H_2O diffusivity
Molecular weight of BMP-4-antibody complex	172 kDa	Molecular weight of BMP-4 + IgG
BMP-4-antibody complex diffusion coefficient in H_2O	6.7371×10^{-7} cm^2/s	Young–Carroad–Bell method[2]
BMP-4-antibody complex diffusion coefficient in PLG scaffold	6.0634×10^{-7} cm^2/s	90% of H_2O diffusivity
TGF-β half-life	26831.5 s (7.45 h)	Exponential curve fit of ELISA data
TGF-β first-order degradation rate	2.583×10^{-5} s^{-1}	$\frac{ln(2)}{half\ life}$
BMP-4 half-life	28037.4 s (7.79 h)	Exponential curve fit of ELISA data
BMP-4 first-order degradation rate	2.472×10^{-5} s^{-1}	$\frac{ln(2)}{half\ life}$
Anti-TGF-β half-life	18,14,400 s (21 days)	Morell *et al.*[3]
Anti-TGF-β first-order degradation rate	3.82026×10^{-7} s^{-1}	$\frac{ln(2)}{half\ life}$
Anti-BMP-4 half-life	18,14,400 s (21 days)	Morell *et al.*[3]
Anti-BMP-4 first-order degradation rate	3.82026×10^{-7} s^{-1}	$\frac{ln(2)}{half\ life}$
TGF-β-antibody complex half-life	18,14,400 s (21 days)	Assumed to be same as free antibody
TGF-β-antibody complex first-order degradation rate	3.82026×10^{-7} s^{-1}	$\frac{ln(2)}{half\ life}$
BMP-4-antibody complex half-life	18,14,400 s (21 days)	Assumed to be same as free antibody
BMP-4-antibody complex first-order degradation rate	3.82026×10^{-7} s^{-1}	$\frac{ln(2)}{half\ life}$
K_D for anti-TGF-β	7.5×10^{-7} g/mL	R&D Systems (Minneapolis, MN)
$k_{on,TGF}$	1467 cm^3/g·s	Calculated from k_{off} and K_D
$k_{on,abTGF}$	8365 cm^3/g·s	Calculated from k_{off} and K_D
$k_{on,TGF\mathrm{complex}}$	9831 cm^3/g·s	Calculated from k_{off} and K_D
$k_{off,TGF}$	1.641×10^{-4} s^{-1}	Stoichiometric equivalent to $k_{off,TGFcomplex}$
$k_{off,abTGF}$	9.359×10^{-4} s^{-1}	Stoichiometric equivalent to $k_{off,TGFcomplex}$
$k_{off,TGFcomplex}$	11×10^{-4} s^{-1}	Yuen *et al.*[4]
K_D for anti-BMP-4	2×10^{-6} g/mL	R&D Systems (Minneapolis, MN)
$k_{on,BMP}$	550 cm^3/g·s	Calculated from k_{off} and K_D
$k_{on,abBMP}$	3088 cm^3/g·s	Calculated from k_{off} and K_D
$k_{on,BMPcomplex}$	3639 cm^3/g·s	Calculated from k_{off} and K_D
$k_{off,BMP}$	1.663×10^{-4} s^{-1}	Stoichiometric equivalent to $k_{off},BMPcomplex$
$k_{off,abBMP}$	9.337×10^{-4} s^{-1}	Stoichiometric equivalent to $k_{off},BMPcomplex$
$k_{off,BMPcomplex}$	11×10^{-4} s^{-1}	Yuen *et al.*[4]

TGF, transforming growth factor; BMP, bone morphogenetic protein; PLG, poly(lactide-co-glycolide); ELISA, enzyme-linked immunosorbent assay.