Supplementary Methods

S1. Detailed study design and regulatory framework

S1.1 Trial classification and regulatory pathway. This study was an exploratory, single-center, first-in-human clinical investigation designed to evaluate the safety, feasibility, and preliminary biological activity of regenerative constructs generated using the BSM1 device. The trial was conducted in accordance with applicable Japanese regulatory guidance issued by the Pharmaceuticals and Medical Devices Agency (PMDA). and relevant national clinical research regulations.

S1.2 Study timeline and visit schedule. The study consisted of a screening/enrollment phase, a subcutaneous embedding phase for in vivo construct generation, and a post-application follow-up phase. At Visit 1 (V1), eligible patients were registered after confirmation of inclusion and exclusion criteria. At Visit 2 (V2), two to four BSM1 devices were surgically implanted subcutaneously. A safety assessment was conducted at Visit 3 (V3), one week after device implantation, to evaluate local and systemic adverse events during the embedding period. At Visit 4 (V4), three weeks after implantation, the devices were surgically retrieved and the regenerated biococktails were applied to the prepared wound sites.

Follow-up visits were conducted at 1, 2, 3, and 4 weeks after application (V5–V8) to assess wound healing progression and safety. Extended follow-up assessments were performed at 8 weeks (V9) and 12 weeks (V10) after application to evaluate clinical outcomes and adverse events within the predefined safety monitoring window.

S1.3 Trial registration and oversight. This investigator-initiated clinical trial was prospectively registered with the Japan Registry of Clinical Trials (jRCT; No. jRCT2032230318) prior to patient enrollment. The study protocol, including predefined primary and secondary endpoints, was approved by the institutional review board of Yokohama General Hospital (approval No. Yokorin 202312) and conducted in accordance with the Declaration of Helsinki and applicable national regulations.

The principal investigator was responsible for study conduct, safety monitoring, and regulatory compliance. Serious adverse events were reported according to institutional and national regulatory requirements.

S2. Eligibility criteria

S2.1 Inclusion Criteria. The full inclusion criteria were as follows:

(1) Age ≥18 years at the time of consent.

(2) Diagnosis of Type 1 or Type 2 diabetes mellitus.

(3) DFUs unresponsive to standard therapy.
(4) Wound, ischemia, and foot infection (WIfI) classification[7]: Wound: 1–3; Ischemia: 0–2; foot Infection: 0–2; and clinical stage 3 or 4.
(5) Expected skin defect of ≥4 cm^2, including the heel area, after debridement; or anticipated need for additional resection of healthy tissue outside and proximal to the wound for wound closure.
(6) Expected survival for ≥12 months and ability to complete a 12-week follow-up after biococktail application.
(7) Written informed consent provided.

S2.2 Exclusion Criteria. The full exclusion criteria were as follows:
(1) Overall condition that made securing the implantation period difficult due to imminent limb amputation or other urgent conditions.
(2) Severe malnutrition (Controlling Nutritional Status [CONUT] score 8–12)[8,9] or severe comorbidities deemed unsuitable for surgical procedures.
(3) Lacking adequate space for implantation of two or more investigational devices due to poor skin condition or a history of exposure to subcutaneous implants.
(4) Underwent invasive surgery within 30 days prior to enrollment, except for procedures related to foot wounds or vascular reconstruction.
(5) Allergy to stainless steel or polyolefin resin.
(6) Uncontrolled diabetes (fasting blood glucose ≥200 mg/dL).
(7) History of, or currently diagnosed with, malignant tumors (except cases with no recurrence or new onset for at least 5 years post-treatment).
(8) Participated in another clinical trial or interventional study within 3 months prior to enrollment.
(9) Previous implantation of the investigational device or previous biosheet application.
(10) Receiving immunosuppressive therapy for autoimmune disease or organ transplantation.
(11) Pregnant or breastfeeding, tested positive on pregnancy tests at eligibility confirmation, or unwilling to use contraception during the trial period.
(12) Ulcers that are unsuitable for adequate debridement (expected wound area ≥25 cm^2 post-debridement).
(13) Severe ischemia causing rapid necrosis progression despite debridement (e.g., severe limb ischemia or vasculitis-induced refractory wounds unsuitable for revascularization).
(14) Expected to undergo major amputation during the trial period due to progressive ulceration, gangrene, or sepsis.
(15) Severe systemic infections (C-reactive protein > 5 mg/dL).
(16) Any patient deemed inappropriate by the investigator for trial participation due to clinical condition or safety concerns.

S3. Study endpoints and outcome definitions

S3.1 Primary endpoint. The primary endpoint of this exploratory study was the successful in vivo formation of autologous regenerative constructs generated through in-body tissue architecture. Successful formation was defined as the retrieval of constructs meeting predefined quality criteria, including macroscopic structural integrity and the presence of pluripotent stem cell–associated cell populations, as assessed by histological and molecular analyses. Constructs that failed to meet these criteria were classified as non-evaluable and were excluded from downstream biological analyses. The primary endpoint was assessed at the time of construct retrieval prior to clinical application.

S3.2 Secondary clinical endpoints. Secondary clinical endpoints were designed to evaluate wound healing outcomes following a single application of the autologous regenerative constructs. These endpoints included longitudinal changes in wound area and wound depth, assessed at predefined follow-up visits using standardized clinical measurement protocols. Clinical severity was further assessed using changes in the Wound, Ischemia, and foot Infection (WIfI) classification. Limb-related outcomes, including the requirement for extensive surgical debridement or amputation, were recorded throughout the follow-up period.

S3.3 Secondary biological endpoints. Secondary biological endpoints focused on characterizing the cellular and molecular features of the in vivo–assembled regenerative constructs. Histological endpoints included the presence and spatial organization of heterogeneous cell populations, extracellular matrix components, and vascular structures consistent with a multicellular regenerative niche. Molecular endpoints were defined by transcriptomic profiles indicative of regenerative signaling pathways, including genes associated with angiogenesis, immune modulation, extracellular matrix remodeling, and growth factor signaling. These biological endpoints were evaluated using excised constructs obtained at the time of clinical application.

S3.4 Safety endpoints. Safety endpoints included the incidence and severity of adverse events occurring during the subcutaneous implantation period and during the 12-week follow-up after construct application. Adverse events were classified as serious or non-serious according to predefined criteria and were further categorized as device-related, procedure-related, or unrelated to the investigational intervention. All serious adverse events and deaths were reviewed for potential causality by the study investigators.

S3.5 Exploratory endpoints. Exploratory endpoints were prespecified to assess potential associations between clinical outcomes and biological characteristics of the regenerative constructs. These included correlative analyses between wound healing trajectories and histological or transcriptomic features of the constructs. Exploratory endpoints were intended to generate hypotheses regarding mechanisms of action and were not used to draw definitive conclusions regarding efficacy.

S4. In-body tissue architecture device and implantation procedure

S4.1 Device structure and materials. The investigational device **BSM1** is composed of an elliptic porous stainless-steel outer cylinder (25 × 13 × 60 mm), a polyolefin resin-based plastic central plate positioned within the cylinder, and two lids. No cells or bioactive agents are incorporated into the device prior to implantation.

S4.2 Subcutaneous implantation protocol. Under general anesthesia, two to four BSM1 devices were implanted subcutaneously by creating pockets in the abdominal or thoracic region. The implantation sites were selected based on anatomical suitability and surgical accessibility. After placement, the devices were secured within the subcutaneous pockets and the incisions were closed according to standard surgical practice.

S4.3 In vivo assembly period and monitoring. Biococktails were generated by subcutaneous embedding of the BSM1 devices for a period of 3 weeks. During the implantation period, the implantation sites were monitored by visual inspection and ultrasonography to assess local tissue responses, including hematoma formation, fluid accumulation or exudation, skin impairment, and signs of allergic or inflammatory reactions.

S4.4 Harvesting procedure and handling. After the in vivo assembly period, the BSM1 devices were surgically retrieved under appropriate anesthesia. The regenerated biococktails were carefully removed from the devices and visually inspected to confirm macroscopic integrity. Constructs were then handled using sterile technique and processed immediately for clinical application or subsequent histological and molecular analyses, as specified in the study protocol. No additional ex vivo manipulation or culture was performed prior to application.

S4.5 Quality control criteria for regenerative constructs. Quality control assessments were performed on all regenerated biococktails prior to downstream analyses or clinical application. Constructs were first evaluated by macroscopic inspection to confirm structural integrity and the absence of visible contamination or mechanical damage. Constructs that failed to maintain

macroscopic integrity were excluded from further evaluation.

In addition, predefined quantitative criteria were applied to ensure consistency across samples. Constructs that did not meet minimum weight requirements were classified as non-evaluable and were excluded from histological and molecular analyses. Quality control criteria were applied uniformly across all cases and were defined prior to study initiation.

S5. Application of regenerative constructs to wound sites

S5.1 Wound bed preparation and debridement. Prior to application of the regenerated biococktails, the wound bed was prepared according to standard surgical and wound care principles. Non-viable tissue was removed through sharp debridement to achieve a clean, well-vascularized wound surface. Hemostasis was achieved before placement of the construct. No additional biologically active agents were applied to the wound bed immediately prior to construct placement.

S5.2 Placement and fixation of constructs. The regenerated biococktails were applied directly to the prepared wound surface in a single procedure. Constructs were positioned to achieve full coverage of the target wound area. When necessary, constructs were secured using standard surgical fixation techniques appropriate for the wound location. No ex vivo modification, expansion, or supplementation of the constructs was performed prior to placement.

S5.3 Post-application wound management. Following application, wounds were managed according to institutional standard care protocols. Protective dressings were applied to maintain a moist wound environment and to prevent mechanical displacement of the construct. Wound sites were monitored at scheduled follow-up visits for signs of infection, inflammation, or graft displacement.

S5.4 Offloading and standard care protocols. Appropriate offloading strategies were implemented for all patients, tailored to ulcer location and patient mobility. Standard care measures included glycemic control optimization, vascular assessment and management when indicated, and infection control according to clinical guidelines. No additional regenerative therapies were administered during the predefined 12-week follow-up period.

S6. Safety monitoring and adverse event classification

S6.1 Definitions of adverse events and serious adverse events. An adverse event (AE) was defined as any untoward medical occurrence observed during the study period, regardless of its causal relationship to the investigational procedure or regenerative construct. A serious adverse

event (SAE) was defined in accordance with international clinical research standards and included events resulting in death, life-threatening conditions, hospitalization or prolongation of hospitalization, persistent or significant disability, or other medically significant conditions.

S6.2 Device-related and procedure-related event definitions. Adverse events were categorized according to their presumed relationship to the investigational intervention.

A device-related event was defined as an adverse event directly attributable to the physical properties, materials, or structural integrity of the BSM1 device, independent of the surgical implantation procedure.

A procedure-related event was defined as an adverse event arising from the surgical implantation or retrieval procedure itself, including events associated with anesthesia, incision, pocket creation, or wound closure, but not attributable to the device materials or structure.

A biococktail-related event was defined as an adverse event occurring after application of the regenerated construct and considered potentially related to its biological properties.

Events were classified as unrelated when an alternative clinical explanation was identified and no temporal or biological plausibility supported a relationship to the device, procedure, or regenerated construct.

S6.3 Safety monitoring during embedding and follow-up. Participants were monitored for adverse events throughout the subcutaneous embedding period and for 12 weeks following application of the regenerated biococktails. Safety assessments included clinical examination of implantation and wound sites, evaluation of systemic symptoms, and review of laboratory and imaging findings where clinically indicated.

S6.4 Causality assessment. All adverse events were reviewed by the study investigators at the time of identification and categorized as device-related, procedure-related, biococktail-related, or unrelated to the investigational intervention. Causality determinations were based on temporal association, clinical evaluation, available laboratory and imaging findings, and assessment of alternative etiologies.

Deaths occurring during the predefined safety monitoring period were evaluated for potential relatedness to the investigational procedure or regenerative constructs. In addition, deaths identified during post-protocol clinical follow-up were documented and reviewed for potential association with the investigational intervention.

S6.5 Reporting and oversight. Serious adverse events were documented and reported in accordance with institutional and regulatory requirements. Safety data were reviewed periodically

to ensure continued appropriateness of study conduct.

S7. Clinical outcome assessments

S7.1 Photographic documentation protocol. Standardized digital photographs were obtained at each study visit using consistent camera positioning, lighting conditions, and imaging distance. A measurement scale was included in every image to allow calibrated digital analysis. Images were archived in a secure database and used for longitudinal comparison and, when necessary, independent verification of wound measurements.

S7.2 Wound area and depth measurement methods. Wound dimensions were assessed at each study visit using standardized digital photographic documentation combined with image-based measurement analysis. All wounds were photographed with a calibrated measurement scale included in the field of view. Maximal length, perpendicular width, surface area, and depth were determined using digital image analysis to ensure objective and reproducible quantification. Measurements were performed according to predefined study procedures by trained clinicians to maintain consistency across visits.

S7.3 WIfI classification and scoring rules. Clinical severity was evaluated using the Society for Vascular Surgery Wound, Ischemia, and foot Infection (WIfI) classification system. Each component (wound extent, ischemia, and infection) was graded according to established criteria. Overall clinical staging was determined based on the composite WIfI stage. All classifications were assigned by clinicians experienced in vascular and wound assessment.

S7.4 Criteria for additional interventions or amputation. Decisions regarding additional surgical debridement, minor amputation, or major amputation were made according to institutional clinical guidelines and based on wound progression, infection control, vascular status, and overall patient condition. Such interventions were recorded prospectively during the study period.

S8. Histological and immunohistochemical analyses

S8.1 Tissue fixation and processing. Excised biococktails were fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), dehydrated through graded alcohols, embedded in paraffin, and sectioned at 4–5 μm thickness for histological analysis.

S8.2 Histological staining protocols. Paraffin sections were deparaffinized, rehydrated, and stained using standard protocols to assess overall tissue architecture and extracellular matrix organization. Masson’s trichrome, Elastica Van Gieson, and Alcian blue staining were performed

to evaluate collagen deposition, elastic fiber distribution, and glycosaminoglycan content, respectively.

S8.3 Immunohistochemistry. For immunohistochemical analysis, deparaffinized sections underwent heat-induced antigen retrieval at 90 °C for 40 minutes. Sections were incubated with primary antibodies overnight at 4 °C, followed by incubation with appropriate secondary antibodies at room temperature. Nuclear counterstaining was performed using DAPI-containing mounting medium.

Primary antibodies targeted markers associated with stem/progenitor cell populations, immune cell subsets, vascular structures, and extracellular matrix components.

S8.4 Image acquisition. Stained sections were examined using a fluorescence microscope equipped with digital imaging capabilities. Representative fields were captured under standardized magnification settings to ensure comparability across samples.

S8.5 Qualitative and quantitative evaluation. Histological evaluation focused on the presence and spatial organization of heterogeneous cellular populations, vascular structures, and extracellular matrix components consistent with multicellular regenerative niche formation. When quantitative analyses were performed, identical threshold and analysis parameters were applied across all samples to ensure consistency. Image assessments were conducted in a blinded or semi-blinded manner where feasible.

S9. Transcriptomic analysis

S9.1 RNA extraction and quality control. Excised regenerative constructs were immediately snap-frozen in liquid nitrogen following surgical retrieval. Total RNA was isolated using phenol-based extraction followed by column purification according to manufacturer protocols. RNA concentration and purity were assessed spectrophotometrically, and RNA integrity was evaluated using an automated electrophoretic system to calculate the RNA Integrity Number (RIN). Samples with insufficient RNA yield or inadequate RNA integrity were excluded from downstream sequencing analysis based on predefined quality criteria.

S9.2 Library preparation and sequencing platform. Ribosomal RNA was depleted prior to library construction to enrich for coding and non-coding transcripts. Strand-specific sequencing libraries were prepared according to validated protocols and subjected to paired-end sequencing (150 bp reads) on a high-throughput sequencing platform. Sequencing was performed by an external certified genomics facility.

S9.3 Data preprocessing and normalization. Raw sequencing reads were subjected to quality filtering and adapter trimming prior to alignment. Filtered reads were mapped to the human reference genome (Genome Reference Consortium Human Build 37). Transcript abundance was quantified at the gene level and normalized as transcripts per million (TPM) to allow comparison across samples.

For each case, normalization and expression profiling were performed independently to enable within-case comparisons between regenerative constructs and matched subcutaneous control tissue. Predefined expression thresholds were applied to exclude low-abundance transcripts from downstream analyses.

Subcutaneous tissue fragments obtained during skin incision were designated as control samples for comparative transcriptomic analyses.

S9.4 Differential expression analysis. Differential expression analyses were conducted to compare regenerative constructs with matched control subcutaneous tissue. Statistical testing was performed using standardized bioinformatics software with predefined significance thresholds established prior to analysis. Where appropriate, correction for multiple testing was applied to control the false discovery rate.

Supplementary Table S1: Test of normality

Area	Shapiro-Wilk		
	Test statistic	Degree of freedom	p-value
V2	0.925	10	0.404
V4	0.949	10	0.656
V8	0.969	10	0.881
V9	0.897	10	0.203
V10	0.711	10	0.001

Depth	Shapiro-Wilk		
	Test statistic	Degree of freedom	p-value
V2	0.897	9	0.233
V4	0.804	9	0.023
V8	0.915	9	0.355
V9	0.937	9	0.554
V10	0.790	9	0.016

Supplementary Table S2: Transcriptomic profiling of in vivo–assembled Biococktails