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Standard Guide for Evaluation of *in vitro* Release of Biomolecules from Biomaterials Scaffolds for TEMPs[1]

This standard is issued under the fixed designation F3142; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 To describe general principles of developing and/or using an *in vitro* assay to evaluate biomolecule release from biomaterials scaffolds for TEMPs, with examples from the literature

1.2 The guide will address scaffolds that do not contain seeded cells; general principles may still apply but may need to be modified if cells are part of the TEMPs.

1.3 *In vitro* release assessment of biomolecules from matrices is a valuable tool for screening biomolecule-scaffold interactions, as well as characterization, and/or quality control.

1.4 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.5 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

F2312 Terminology Relating to Tissue Engineered Medical Products

F2809 Terminology Relating to Medical and Surgical Materials and Devices

F2902 Guide for Assessment of Absorbable Polymeric Implants

2.2 *United States Pharmacopeia (USP) General Chapters:*[3]

USP<1> Injections and Implanted Drug Products (Parenterals)—Product Quality Tests

USP <711> Dissolution

USP <724> Drug Release

USP <785> Osmolality and Osmolarity

USP <1092> The Dissolution Procedure: Development and Validation

2.3 *European Pharmacopeia (Ph. Eur.):*[4]

Ph. Eur. 2.9.3 Dissolution Test for Solid Dosage Forms

Ph. Eur. 2.9.4 Dissolution Test for Transdermal Patches

Ph. Eur. 2.9.25 Dissolution Test for Medicated Chewing Gums

2.4 *FDA Document:*[5]

FDA Guidance for Industry Bioanalytical Method Validation (2001)

3. Terminology

3.1 The present document uses the definitions of Terminologies F2809 and F2312.

4. Significance and Use

4.1 The European Pharmacopoeia (Ph. Eur.) as well as the United States Pharmacopeia (USP) describe several dissolution and drug release setups for tablets, capsules, transdermal patches and suppositories (USP <711>, USP <724>, Ph. Eur. 2.9.3, Ph. Eur. 2.9.4). However, up to this point no pharmacopoeia standardized *in-vitro* release test has been established for parenteral dosage forms which provide sustained drug release, for example, implants.

4.2 An appropriately designed *in-vitro* release test would be favorable in the early stage of development of biomolecule-releasing scaffolds for TEMPs, as well as in quality control, and may help to reduce the number of animal experiments.

4.3 Appendix X1 provides a tabulated overview of published *in-vitro* release studies performed with biomaterial scaffolds loaded with biomolecules.

4.4 One goal of *in-vitro* release studies is to simulate the *in-vivo* conditions as closely as possible, but with sufficiently

[1] This test method is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from U.S. Pharmacopeial Convention (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

[4] Available from EDQM Council of Europe, 7 allée Kastner, CS 30026, F-67081 Strasbourg, France, or visit the website, www.edqm.eu.

[5] Available from Drug Information Branch (HDF-210), Center for Drug Evaluation and Research (CDER), 5600 Fishers Lane, Rockville, MD 20857, or visit the website, http://www.fda.gov/cder/guidance/index.

simplifying abstraction. The simplification comprises two general aspects: the amount of fluid or release medium in contact with the implant to simulate the physiological environment, and the composition of that release medium.

5. Elements of *in vitro* Release Assays

5.1 *Sample (biomaterial scaffold loaded with biomolecule(s)):*

5.1.1 The sample should be taken from the final product, manufactured by a representative process including packaging and sterilization.

5.1.2 There are several considerations that will influence the selection of the sample dimensions.

5.1.2.1 Vessel size into which the sample can physically fit.

5.1.2.2 Method used to generate a representative sample, where needed, for example, by biopsy punch, cutting with a scalpel blade.

5.1.2.3 Load of the biomolecule, and the concentration in the final setup.

5.2 *Release Medium:*

5.2.1 In order to simulate physiologic conditions at the intended implant site, the release medium should be of appropriate tonicity (osmolality) and pH. Regarding osmolality, the term is used according to USP <785> and as such is a measure of concentration of real solutes, expressed in osmole per [kg] of soluent. Isotonic solutions are iso-osmotic relative to whole blood. Isotonicity is a target attribute for parenteral injections per USP <1>.

NOTE 1—Cells, for example red blood cells, are susceptible to conditions that are outside the isotonic range, by showing swelling through influx of water through the cell membrane (potentially leading to burst) when in a hypotonic environment, or shrinking through efflux of water through the cell membrane when in a hypertonic environment. Regarding pH, the medium should be buffered to prevent pH shifts over time due to the release of the biomolecule, and/or degradation of the matrix. pH shifts could change the release mechanism or kinetics.

5.2.2 One medium used very often in the published literature is phosphate-buffered saline (PBS), pH 7.2 or 7.4, in the range of isotonicity (approximately 300 mOsm/kg). In this case, the buffer system is provided by phosphate salts; the tonicity is typically augmented with sodium chloride.

5.2.3 The release medium may be spiked with other components to make it more similar to serum, for example, bovine serum albumin (BSA), or fetal bovine serum (FBS; multicomponent additive). It should be noted that these additives can substantially interfere with the detection method (see 5.7). If protein components are added to the medium (BSA, FBS), evaluation of *in vitro* release will require more specific and/or sophisticated assays (compared to simple A280 UV detection for concentration), as both the biomolecule that is studied, and the proteinaceous component(s) of the medium may contribute to the detected signal. The same applies if there are several biomolecules that can be released from the biomaterial scaffold. Further considerations are described in 5.8 (especially 5.8.4 and 5.8.6). It should also be noted that regarding the use of BSA and FBS, it is important to limit the use to a single or limited number of lots to reduce variability in the assay system, especially for studies that require more than a single iteration.

5.2.4 One of the important factors is to determine if there are any solubility issues for the biomolecule, in order to define the most appropriate medium. This primary evaluation can be achieved by exposing the biomolecule to the same experimental conditions without the scaffold present, to create a baseline in the candidate medium, and comparing it to a known stable formulation of the biomolecule at the same concentration levels. If the concentrations measured in the candidate medium are substantially the same as in the known stable formulation, it is a good indicator for sufficient solubility and minimum absorption to surfaces in the system (see also 5.3.5).

5.2.5 If the experiment is set up for an extended period of time to simulate the target period of release *in vivo* (that is, several days to weeks or months), consider adding azides or other preservatives to maintain the sterility of the medium and aseptic conditions in the setup. Possible impact of any additive on either the sample or the analytical method should be evaluated.

5.2.6 The ratio of the volume of release medium to biomolecule load (which may correspond to the volume or size of the construct) is important. If there is a requirement to have sink conditions in the experiment (for example, to simulate implantation sites with high fluid circulation), then sink conditions can be achieved by using a large volume of release medium at the beginning of the experiment and extraction of small aliquots, or frequent exchange of the medium. Per USP definition, sink conditions can be maintained at about three times the volume of a saturated solution, and it has been suggested that it should remain below 10 % of saturation concentration in the solution (Reference: USP <1092>). As indicated in 5.2.4, and 5.8.8, the saturation concentration in the candidate medium may need to be experimentally determined.

NOTE 2—Solubility of biomolecules is dependent on pH, salts, and temperature, and may vary widely not only between biomolecules but also for each biomolecule at different conditions (for example, BSA reported to be soluble in water at 40 mg/mL; vendor data sheets for basic fibroblast growth factor (bFGF), nerve growth factor (NGF), and bone morphogenetic protein-2 (BMP-2) recommend reconstitution of lyophilized material to not less than 100ug/mL. Note that lyophilized material is typically formulated and contains some buffer/salt/stabilizers).

5.2.7 The addition of a protease or other enzyme may be considered, if the biomolecule is expected to be tightly bound or incorporated. The presence of the protease or other enzyme may interfere with the assay used to quantify the biomolecule of interest (see 5.2.3). Also, the susceptibility of the biomolecule of interest to the protease or other enzyme must be experimentally evaluated.

5.2.8 5.2.8 In cases where the biomolecule is tightly bound or incorporated, harsher extraction media may be required. For example, for extraction of BMP-2 from bone matrix, buffers containing urea (Urist 1984 (**1**))[6], ethylene glycol (Urist 1979 (**2**)), or an arginine-histidine combination (Hollinger 1996 (**3**)) have been described.

5.3 *Vessel/Container:*

5.3.1 The vessel needs to be of appropriate size to contain the sample as well as the medium.

[6] The boldface numbers in parentheses refer to a list of references at the end of this standard.

5.3.2 The vessel needs to have a tight closure, and tight connections in any fluid paths, to keep the system intact. The main concerns are microbiological contamination, but also evaporation which could impact concentration.

5.3.3 USP <711> and USP <724> describe several vessel configurations that may be appropriate and commercially available to evaluate *in vitro* release of biomolecules from biomaterials scaffolds for TEMPs.

5.3.4 Vessels may be flow-through cells, or static vessels with internal, external, or no mixing mechanism. Mixing versus static conditions are discussed further in 5.5.

5.3.5 The surface and contact materials should be evaluated regarding adsorption or absorption characteristics of the biomolecule at the concentrations expected in the experiment. Adsorption/absorption phenomena could have an effect on concentration results, as well as the structural integrity of the molecule.

5.4 *Temperature-Controlled Environment:*

5.4.1 The experimental condition most often used is 37°C, which corresponds to commonly accepted human core temperature. Other temperatures may be appropriate for specific applications or clinical conditions.

5.4.2 Temperature-controlled configurations described in USP <711> and USP <724> may be appropriate and are commercially available. Alternatively, appropriate vessels may be placed in incubators or a water bath. Wrap-around temperature jackets may be appropriate for larger vessels.

5.4.3 Increasing the temperature to accelerate release may or may not be appropriate, as the temperature may change the characteristics or impact the integrity of the scaffold, and thus alter the release mechanism (see Guide F2902). Temperature also may impact the integrity of the biomolecule.

5.5 *Static Conditions versus Dynamic Conditions:*

5.5.1 The dissolution methods described in the USP are adding a stirring movement or flow-through to facilitate transport and distribution from the surface and immediate vicinity of the dosage form. A static system *in vitro* would likely also not be reflective of the *in vivo* situation for TEMPs, although it might be difficult to define a universal fluid flow simulating all anatomic sites and conditions.

5.5.2 The physiologic condition at the most likely implantation scenario should be considered, for example, swelling after surgery with considerable influx of fluid.

5.5.3 Ph. Eur. 2.9.25, dissolution test for medicated chewing gums adds a mechanical stress to simulate the chewing motion. While the standard frequency (described as 60 per minute) may not be completely appropriate for TEMPs, the general principle and apparatus might be worth considering in the case where the implantation site, and thus the TEMPs construct, may be exposed to muscle movement.

5.6 *Sampling Mechanism:*

5.6.1 Options of pulling samples may be linked to or even defined by the vessel and general experimental setup, as well as the assays conducted.

5.6.2 In general, the sample pulled may require some separation of the TEMPs from the release medium. This can be achieved by sampling from the surface or a location remote from the construct to avoid accidentally removing parts of the construct. Alternatively, separation of the releasing construct from the bulk of the medium has been achieved by placing the construct inside a dialysis membrane, or reverse dialysis (construct in the bulk of the medium, samples pulled from the dialysis cassette).

5.6.3 Options include manual sampling, or automation. Automation may decrease the risk of accidental contamination during sample pull, as well as operator errors (for example, pulled versus replaced volume, timing, etc.). Some setups may offer the option of in-line measurements in a circulating system.

5.7 *Sampling Time Points and Total Time:*

5.7.1 The frequency of sampling can influence the release by creating sink conditions versus no-sink conditions. As mentioned above, sink conditions are maintained when the volume exceeds approximately three times the saturation concentration. Frequent sampling (with replenishing), or sampling a large amount of the medium at a lower frequency, is conducive to remaining far below saturation levels.

5.7.2 Sampling time points should also take into consideration the stability of the analyte. A theoretical decrease in biomolecule detection can be introduced due to the stability of the released analyte, or changes to the analyte (see also 5.8.9.)

5.7.3 The frequency of sampling should be matched with detectability in the assay (especially if the sample volume is replaced after sampling, there is a constant dilution). Limit of quantitation (LOQ) and limit of detection (LOD) should be understood. Fig. 1 illustrates the theoretical decrease in biomolecule concentration in the release medium due to removing aliquots during sample pull, using a 10 % and 66 % sample volume exchange rate at each time point. At 66 % exchange, the initial total amount of the biomolecule in the system would be reduced to 10 % after only 2 time points.

5.7.4 Storage of release medium aliquots (condition and length of time before assaying) should be amenable to the biomolecule. Storage vials should be assessed for adsorption, similar to the description in section 5.3.5.

5.8 *Assay(s):*

5.8.1 Careful consideration needs to be given to the selection of the analytical assay(s).

5.8.2 Literature review around characterization of parenteral drug delivery systems and biomaterial scaffolds containing biomolecules revealed that the majority of *in vitro* release experiments have been performed either with radioactively labeled compounds or by using immunosorbent-linked assays for detection, or studying cell-based effects.

5.8.3 The first approach requires facilities where radioactive material can be handled, and may not be feasible for final product characterization for unitary products, as it requires labeling prior to combination with carrier materials. This in itself could possibly lead to changes in protein/carrier interaction. Immunosorbent-linked assays may be difficult to realize with high amounts of samples. For an industry laboratory, it might be desirable to have an *in vitro* release assay where the biomolecule can be analyzed by standard ultraviolet (UV) light spectroscopy or high performance liquid chromatography (HPLC) methods and does not have to be labeled.

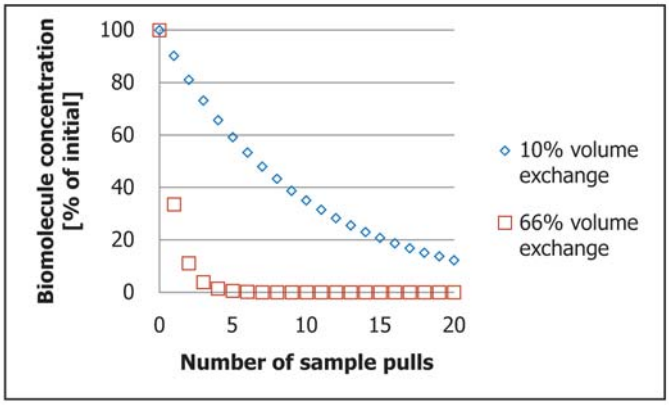

FIG. 1 Calculated Decrease in Biomolecule Concentration in the Release Medium Due to Removing Aliquots During Sample Pull

5.8.4 If there are several biomolecules that can be released from the same biomaterial scaffold, the methods will need to be evaluated for cross-reactivity.

5.8.5 The first decision is whether the goal is only quantitation of the biomolecule, versus determining or proving integrity of the biomolecule (via bioassay and other evaluation techniques, for example, to study primary and/or higher structure).

5.8.6 The sensitivity of the analytical method(s) needs to be determined (LOQ, LOD; see 5.7.3.). This needs to be determined in the presence of potentially interfering components from the matrix, as well as from the medium.

5.8.7 For quantitation, it is necessary to establish a linear correlation, or alternatively a known, reproducible correlation (concentration response). According to FDA Guidance for Industry, the simplest model that adequately describes the concentration-response relationship should be used. Selection of weighting and use of a complex regression equation should be justified. Values of samples in the calibration curve should show not more than a 15 % deviation from the nominal value (20 % for the lower limit of quantitation).

5.8.8 It is necessary to understand the solubility of the biomolecule (protein) in the medium, which will determine the medium/sample ratio of the setup and the analytical requirements.

5.8.9 Stability of the protein under the test conditions (for example, 37°C, pH, required time, addition of preservative) needs to be evaluated and well understood, in order to determine the contribution of the scaffold to *in vitro* release. It may be advisable to run a control sample to establish the nominal 100 % release result in each evaluation (that is, biomolecule load in the medium without the carrier matrix, without any sample pulls.). If the scaffold can contribute a background signal in the selected assay, it is advisable to include a control sample of the biomolecule-free scaffold in the release medium in the experiment.

5.8.10 It should be determined if the selected assays are compatible with the medium, or if additional sample preparation is required, for example, to adjust concentration (example: cell-based bioassays may need further dilution in a different medium, which can create a limit to the lowest concentration possible in the release medium.)

5.8.11 If additional sample preparation (for example, centrifugation, filtration, dialysis) is needed, the stability of the biomolecule throughout the process needs to be evaluated.

5.8.12 See FDA Guidance for Industry or current version for guiding principles around validating assays for regarding selectivity, accuracy, precision, recovery, stability, linearity, etc. The assay needs to be validated in the final system, that is, for the specific biomolecule(s) in the context of the specific biomaterial scaffold.

5.9 As shown in the previous subsections, *in vitro* release evaluation involves understanding of a complex system. The inter-relations of sample size, medium, and the analytics are shown in Fig. 2 for illustration. For example:

5.9.1 The lower the carrier volume, or more specifically, the biomolecule load, the more sensitive the detection method needs to be.

5.9.2 The higher the release medium volume, the more sensitive the detection method needs to be.

5.9.3 The exchange rate of the medium, that is, both the frequency of sample pull and the % volume exchanged, will impact the concentration of the biomolecule in the system, and thus require a sensitive detection method.

5.9.4 The release medium, specifically any proteinaceous additives, will impact the detection method, as a higher specificity is needed. (In Appendix X1, this is reflected in ELISA and bioassay methodology typically being used for media containing serum (FBS), or BSA).

5.9.5 If the carrier matrix is proteinaceous (for example, collagen or gelatin-based), a highly specific detection method is needed for accurate evaluation of *in vitro* release of the biomolecule from the TEMPs scaffold.

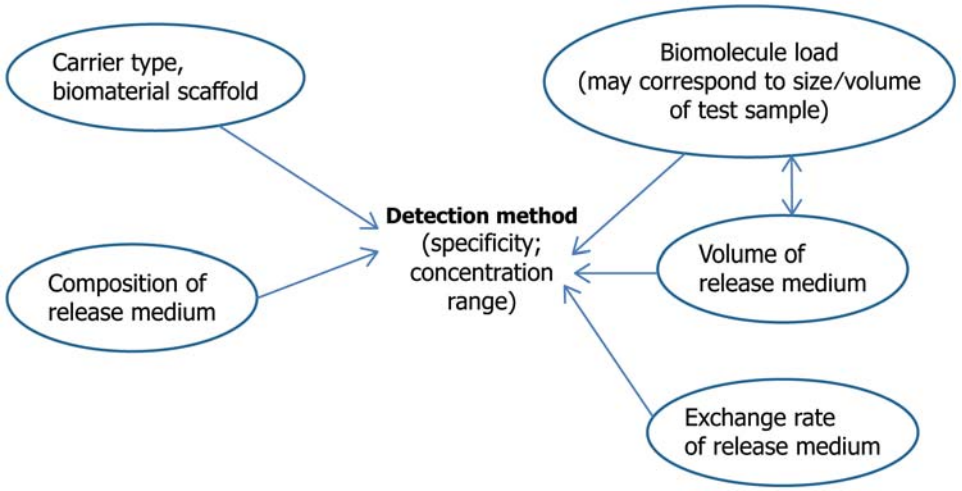

FIG. 2 Interrelationship of Factors in *in vitro* Release Experiments

APPENDIX

(Nonmandatory Information)

X1. PUBLISHED *in-vitro* RELEASE STUDIES PERFORMED WITH BIOMATERIAL SCAFFOLDS LOADED WITH BIOMOLECULES

X1.1 Selection of studies on *in vitro* release from biomolecule/biomaterial scaffold combinations (Table X1.1):

TABLE X1.1 Overview Table
(alphabetical order by biomolecule name)

Protein	Carrier	Release Medium and Setup	Analytical Method	Reference
bFGF	Collagen films, heparinized	5 mL cell culture medium (5 % human serum)	^{125}I, ELISA	Wissink 2001 (4)
bFGF	Collagen films	Cell culture medium (5 % human serum)	ELISA	Wissink 2000 (5)
bFGF	Gelatin	PBS	Fluorescence-labeled, HPLC	Tabata 1998 (6)
bFGF; BMP-2	PLGA cylinders, microspheres; with $Mg(OH)_2$/BSA	PBS + BSA + heparin	Bradford protein assay; BIACORE (anti-BMP fixed)	Zhu 2000 (7)
bFGF	poly(ester urethane)urea scaffolds	DNEM containing 0.5 % FBS and 1 % pen/strep	^{125}I; rat vascular smooth muscle cell proliferation	Guan 2007 (8)
rhbFGF	chitosan-alginate scaffolds	PBS	ELISA; Hs68 cell proliferation	Ho 2009 (9)
bFGF	chitosan-hydroxyapatite scaffolds	PBS with 0.1 % azide	Fluorescence spectrophotometer (intrinsic tryptophan fluorescence)	Tigli 2009 (10)
BMP-2; BMP-4	Gelatin films (GTA-xl)	Cell culture medium	Bioassay (C3H10T1/2 — ALP activity; DNA content)	Raiche 2001 (11)
rhBMP-2	Collagen sponge, hyaluronic acid matrix, ceramics	Bovine serum, 100 % exchange	^{125}I	D'Augusta 2000 (12)
rhBMP-2	PLGA microspheres	PBS pH 7.4 (isotonic, or 0.1 M resp.)	RP-HPLC	Druggirala 1966a,b (13, 14)
rhBMP-2	Poly (D, L-lactide) disks	Cell culture medium (5 % FBS)	Bioassay (OPCI cells — ALP activity)	Winn 1999 (15)
rhBMP-2	PLGA-hydroxyapatite scaffolds	PBS	ELISA; also used continuous Native-PAGE to determine integrity and conformation of eluted BMP-2	Nie 2008 (16)
rhBMP-2	Dextran-derived microspheres	PBS	^{125}I; determined BMP activity using human periodontal ligament cells (proliferation, ALP, osteocalcin, and osteopontin)	Chen 2006 (17)
rhBMP-2	PEG-PLGA microspheres	PBS with 0.05 % polysorbate 20, 1 % BSA, 0.02 % axide, and 0.15 % EDTA	ELISA; determined BMP activity using C2C12 mouse myoblasts (ALP)	Lochmann 2010 (18)
rhBMP-2	PLGA scaffolds	DMEM containing 10 % FBS	ELISA; determined BMP activity using rabbit MSCs (proliferation, ALP, collagen, and mineralization)	Liu 2007 (19)
rhMBP-2	PLGA microspheres in polyurenthane scaffolds	Cell medium containing 1 % BSA	ELISA; determined BMP activity using MC3T3 cells (ALP)	Li 2009 (20)
rhBMP-2	Hydroxyapatite-β-TCP	DMEM containing 10 % FBS and 1 % pen/strep	^{125}I	Autefage 2009 (21)
Bovine calcitonin	microparticles: PLGA, PET-PLGA	PBS with polysorbate; tube versus dialysis bags (sink)	^{125}I	Diaz 1999 (22)
BSA	PLGA scaffold	PBS (sink)	Fluoraldehyde assay (Pierce)	Whang 2000 (23)
BSA; sp55R	PLGA	PBS	UV (280 nm); ELISA	Eliaz 2000 (24)
BSA, insulin	Starch microspheres	Phosphate buffer (pH=6.4) containing 3 mM azide, 185 U/L α-amylase, and 0.1 % Poloxamer NF 188	HPLC	Elfstrand 2009 (25)
BSA, FGF-1, FGF-2, VEGF-165, heparin-binding EGF	Chitosan hydrogels	PBS	BSA-BCA protein assay FGF-1, FGF-2, VEGF-165, and HB-EGF-ELISA	Ishihara 2003 (26)
BSA; multitude of growth factors	Gelatin hydrogel films (dextran-dialdehyde-xl)	PBS + 0.1 % casein; films mounted on cell culture insects in culture plates	^{125}I	Draye 1998
hEGF	Chitosan gel	Phosphate buffer (pH=5.8) with samples in dialysis tubing	Spectrofluorometer	Alemdaroglu 2006 (27)
Growth hormone	Collagen/HA/PVA sponges	PBS (complete removal)	ELISA	Cascone 1994 (28)
rhHGF (hepatocyte growth factor)	Gelatin hydrogels	PBS	^{125}I	Ozeki 2006 (29)
HGF and BSA	PHBV/PLGA microspheres scaffolds	BSA-PBS HGF-serum-free media	BSA-HPLC HGF-EISA	Zhu 2009 (30)
Horseradish peroxidase	PLLA-PDGLA	Double-distilled water containing 0.05 % (w/w) azide	BCA protein assay	Ziberman 2007 (31)
rhIGF-I	PLGA microspheres	PBS containing 5 mM SDS, 0.1 % BSA, 01 % BSA, and 0.1 azide; horizontal shaking	Radioimmunoassay (RIA); MG-63 cell proliferation	Meinel 2003 (32)
modified IGF-I	Fibrin gel	PBS containing 0.1 % BSA and 1 % pen/strep	ELISA	Lorentz 2012 (33)
rhIGF-I	PLGA microparticles	PBS	RP-HPLC	Singh 2001 (34)
IFN	collagen minipellet	PBS + 0.05 % HSA	RIA	Fujioka 1995 (35)
Insulin	No carrier: suspension	PBS, flow-through	UV (214 nm)	Graham 1984 (36)
rhNell-1	Alginate-chitosan particles	PBS	CBQCA protein assay (better detection limit than BCA and micro-BCA assays)	Lee 2009 (37)

TABLE X1.1 *Continued*

Protein	Carrier	Release Medium and Setup	Analytical Method	Reference
NGF (nerve growth factor)	Alginate/chitosan tubes coated with PLGA	citrate-buffered saline (pH=5.0) or acetate-buffered saline (pH=5.5) with 0.05 % Tween 20; authors claim this media helps NGF remain active	ELISA; PC12 cell differentiation	Pfster 2008 (38)
NGF	pHEMA hydrogels	PBS; then dialyzed sample against sodium borate buffer to run CBQCA protein quantification assay	CBQCA protein quantification assay, used NGF to make standard curve	Jhaveri 2009 (39)
PRP-derived growth factors (PDGF-AB, TGF-β1, IFG-1	Alginate	DMEM	ELISA	Lu 2008 (40)
Protein A	CPC	PBS	Protein A labeled with FITC-measured fluorescence	Xu 2008 (41)
rhPDGF-BB	Chitosan/TCP sponge	PBS	^{125}I	Lee 2000 (42)
PDGF-BB	Chitosan sponge	PBS	^{125}I	Park 2000 (43)
rhbFGF	PLGA foams	PBS	Bradford protein assay; bioassay (fibroblasts)	Hile 2000 (44)
Tetanus toxoid	PLGA microspheres	Layer system: (Agarose gel + protein) + agarose gel + PBS	ELISA	Johansen 1998 (45)
TGF-beta	Lipid-based mocrocylinders	PBS	^{125}I; bioassay (mink lung cells)	Spargo 1997 (46)
TGF-beta 1	Porous glass carrier	PBS + 1 % BSA	Bioassay (mink lung cells)	Nicoll 1997 (47)
TGF-β1	Collagen-chitosan microgranules	PBS	^{125}I; MC3T3–E1 cell proliferation	Lee 2006 (48)
TGF-β1	PLGA microparticles	1 % BSA	ELISA; hMSC cell proliferation	Clark 2008 (49)
TGF-β1	Chitosan-starch-β-glycerol phosphate hydrogel	DMEM containing 10 % FBS and pen/strep	ELISA	Faikrua 2012 (50)
TGF-β3	Alginate microspheres in hyaluronic acid hydrogels	Cell culture medium; containers pre-treated with 1 % BSA to minimize protein adsorption	ELISA	Bian 2011 (51)
rhTGF-β3	PLGA microspheres	1 % BSA	ELISA	Moioli 2006 (52)
TGF-β1, IFG-1	Gelatine microparticles in oligo(poly(ethylene glycol) fumarate) hydrogels	PBS with collagenase	^{125}I	Holland 2005 (53)
TGF-β2 + BSA	EVA rods	PBS	Biorad protein assay; ELISA for TGF-β	Kim 1997 (54)
TI (trypsin inhibitor)	Silica xerogels	Tris-(hydroxymethyl)-aminomethane buffer pH 7.3, total exchange	Gold colloidal assay	Santos 1999 (55)
VEGF	PLGA scaffolds	SBF, PBS	^{125}I; retention	Murphy 2000 (56)
VEGF, PDGF	Chitosan-brushite	DMEM containing 2 % FBS and 0.2 % azide	^{125}I	De la Riva 2010 (57)
VEGF	PLA, PLGA microspheres	Cell culture medium (serum-free or 10 % FBS)	^{125}I, bioassay	Eiselt 1998 (58)
rhVEGF-121	Alginate hydrogels coated with chitosan and dextran sulfate	PBS	ELISA	Matsusaki 2007 (59)
rhVEGF165, rhBMP-2	Alginate-PLA scaffolds	PBS	ELISA; determined BMP-2 activity using C2C12 cells (ALP); determined VEGF activity using HUVEC cells (proliferation)	Kanczler 2010 (60)
VEGF, BMP-2	Gelatin microparticles in porous scaffolds	PBS with 400 ng/mL collagenase 1A	^{125}I	Young 2009 (61)
VEGF, Angiopoeietin-I	PLGA microspheres in alginate hydrogel	DPBS	ELISA assay kit	Shin 2013 (62)
VEGF	Electrospum gelatin mats (crosslinked)	HBSS with 0.1 % BSA	ELISA assay kit	Del Gaudio 2013 (63)
Mimetic VEGF, modular VEGF, modular BMP-2	Hydroxyapatite-coated PCL	DMEM	VEGF labeled with carboxyfluorescein, BMP labeled with rhodamine-measured fluorescence	Suarez-Gonzalez 2012 (64)
Zn-rHir; insulin	No carrier — suspension!	Layer system: (Agarose gel + protein) + agarose gel + PBS/ HEPES/serum	Biorad protein assay, fluorescence spectroscopy FOBIA (immuno-assay)	Gietz 1998 (65)

TABLE X1.1 *Continued*

Protein	Carrier	Release Medium and Setup	Analytical Method	Reference
ALP, alkaline phosphatase; BCA, bicinchoninic acid; bFGF, basic fibroblast growth factor; β-TCP, β-tricalcium phosphate; BMP, bone morphogenetic protein; BMP-2, bone morphogenetic protein-2; BMP-4, bone morphogenetic protein-4; BSA, bovine serum albumin; DBQCA, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde; DMEM, Dulbecco's modified eagle medium; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; ELISA, enzyme-linked-immunosorbent assay; EVA, ethylene vinyl acetate; FBS, fetal bovine serum, FGF-1, fibroblast growth factor-1, FGF-2, fibroblast growth factor-2; FOBIA, fiber optic biospecific interaction analysis; GTA-xl, glutaraldehyde crosslinked, HA, hydroxyapatite; HB-EGE, heparin-binding epidermal growth factor-like growth factor; HBSS, Hank's balanced salt solution; HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); HGF, hepatocyte growth factor; hMSC, human mesenchymal stem cell; HPLC, high performance liquid chromatography; HUVEC, human umbilical vein endothelial cells; I, iodine; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor, PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PCL, poly(e-caprolactone); PDGF, platelet derived growth factor; PDGF-AB, platelet derived growth factor-AB; PDGF-BB, platelet derived growth factor-BB; PDGLA, poly(D-glycolic-lactic acid); PPF, poly(propylene fumarate); PLA, poly(lactic acid), PEG, poly(ethylene glycol); PHBV, poly(3-hydroxybutyrate-co-3-hydroxyvalerate); pHEMA, poly(2-hydroxyethyl methacrylate); PLGA, poly(lactic-co-glycolic acid); PLLA, poly(L-lactic acid); PRP, platelet rich plasma; PVA, polyvinyl alcohol; rhBMP-2, recombinant human bone morphogenetic protein-2; rhbFGF, recombinant human basic fibroblast growth factor; rhHGF, recombinant human hepatocyte growth factor; rhIGF-1, recombinant human insulin-like growth factor-1; rhNell-1, recombinant human NELL-1; rhPDGF-BB, recombinant human platelet derived growth factor-BB; rhTGF-β3, recombinant human transforming growth factor-β3; RP-HPLC, reverse phase high performance liquid chromatography, rhVEGF, recombinant human vascular endothelial growth factor; SBF, simulated body fluid; TGF, transforming growth factor; TGF-β, transforming growth factor-β; TGF-β1, transforming growth factor-β1; TGF-β2, transforming growth factor-β2; TGF-β3, transforming growth factor-β3; UV, ultraviolet; VEGF, vascular endothelial growth factor				

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