Loughborough University	Safety Department use only		Material(s) Classification	
	Reference Number:		Hazard Group 1	☐
			Hazard Group 2	☑
Biological Risk Assessment	CBE Use only		GMO	☐
	Reference Number:	CBE 167	HTA Licensable	☐

FORM CBE-RA-Form/002 Version 0.3

RISK ASSESSMENT AND PROJECT REGISTRATION FOR WORK INVOLVING BIOLOGICAL MATERIAL

PLEASE READ CAREFULLY

This form acts to register projects involving the use of Biological Agents and / or Genetically Modified Micro-Organisms, or of materials that may be contaminated with these agents. It assesses the hazards and risks associated with the project as well as identifying those at risk and the measures necessary for preventing, or controlling these risks. Please ensure that sufficient detail is provided when completing this form and that the relevant written SOPs are referenced where required. Once completed and approved, all risk assessments must be supplied to all those working within this project. The work described within this form must not commence until this risk assessment has been completed and approved and that all necessary control measures are in place.

Any changes to the work, or the persons involved, must be notified to the authorised person. All changes requested must be recorded within the risk assessment change control form and may also need to be incorporated within an amended version of this form.

A separate risk assessment will be required for assessing risks associated with GMO activities.

The following declaration must be completed and undersigned by the Principal Investigator or Person Responsible for the project

- All information contained in this form is accurate and comprehensive.
- All workers involved will be instructed that their work must remain within the boundaries of this project registration & assessment.
- All workers have been given, or will be given before they become involved, adequate training and where necessary their competency assessed.
- All workers have, or will be before their involvement begins, enrolled with Occupational Health for health clearance where necessary.
- It is understood that this risk assessment shall not be transferred to a third party without the PI/Supervisor/Line Manager named in this form either taking responsibility for the new activities, or ensuring that a new proposal is submitted.
- All changes to the work covered by this form will be reassessed & the changes submitted to the authorised person before those changes are made to the work.

Principal Investigator		Person conducting this risk assessment	
Name	Elizabeth Ratcliffe	Name	Jenna Davis
Position	Vice Chancellors lecturer in Biological Engineering	Position	PhD student
Department	Chemical Engineering	Department	Chemical Engineering
School	AACME	School	AACME

The Project Activity			
Title	Fish oils as antimicrobials against the bacteria types often found present in diabetic foot ulcers		
Reference Number	000		
Start Date	15 Oct 2018	End Date	10 Sep 2021

Others involved in the work	
Names	Martin Lindley
	Angharad Evans

Name	E. RATCLIFFE	Signature	E. Ratcliffe	Date	3/10/18

1. INTRODUCTION

1.1 Background & aim of project	Assessing the components of food grade fish oils and their derivatives (EPA, DHA and resolvins) when used in different combinations against bacterial species often indentified in biofilm formations on diabetic foot ulcers. The combined effect of introducing aspirin and non-steroidal anti-inflammatory drugs will also be investigated. The project will utilise biofilm assayd and 2D wound scratch models
1.2 Description of experimental procedures	Bacterial growth and killing assays will be performed. Bacteria will be cultured and subjected to fish oils before the level of bactericide will be measured. Assay methods are to be developed fully during the project, but should involve culture of a biofilm on a cover slip and subjecting it to fish oils. Bacteria to be used are all Hg2. If any new bacteria are used that are not listed in the assessment they will be added by the risk assessment review procedure. A further 2D scratch model will be performed (Chen et al. 2014 is attached for methods), where in cells are cultured and a line scratched through with a pipette tip to study the regrowth of cells.
1.3 Where will this work be carried out?	Rooms/areas: H23, H25 and H29
	Building(s): CBE

☑ **2.1 Human or animal tissues, cells, body fluids or excreta will be used in this project**

2. TISSUES, CELLS, BODY FLUIDS OR EXCRETA

2.2 List all cells, tissues, body fluids and excreta to be used. For cells, indicate primary, continuous or finite.

Material type	Organ source	Species	Where it will be obtained from (include country of origin)
Human neonatal dermal fibroblasts and adult epidermal keratinocytes	Skin	Human	ATCC

☑ **2.3 Material(s) listed in section 2.2 above are considered to be 'relevant material' under the Human Tissue Act 2004.**

2.3.1 Relevant material type	Source / Provider *A = Commercial provider* *B = HTA licensed Biobank with REC approval for genetic research use* *C = Other* *D = Organisation with REC approval for research use* *E = Imported*					
Fibroblasts and keratinocytes	☑ A	☐ B	☐ C	☐ D	☐ E	ATCC

2.4 Has any material listed in 2.2 been genetically modified in any way? If Yes, add a reference number and complete the GMO Risk Assessment Form.	○ Yes ⊘ No
☐ 2.5 Has any of the material listed in section 2.2 been identified in the list of cross-contaminated / misidentified cell lines?	○ Yes ⊘ No
2.6. Describe what infectious/communicable disease agents or diseases this material(s) has been screened for, eg HIV, HBV, HCV, TSEs, HTLV etc. *If Yes, provide details*	⊘ Yes ○ No
☐ 2.7. Will any clinical history or veterinary screening be provided?	
2.8 What is the likelihood of infection of any of this material? *Consider the worst case if multiple materials are to be used.*	The risk is: ○ High ⊘ Low ○ Medium ○ None
2.9 Name and classify the biological agents this material could be infected with	Material Type:
	Agent:
	ACDP / Defra Classification:
2.10 Describe the type and severity of the disease that can be caused to humans or animals by each of the agents that could be present	

☑ 2.11 Biological agents will be used in this project

2. BIOLOGICAL AGENTS (i.e. micro-organisms such as bacteria, fungi, microscopic endoparasites)

2.12 List the biological agents to be used	Name of Agent	Strain(s)	ACDP / Defra Classification
	Escherichia coli	ATCC25922	Hazard Group 2
	Staphylococcus aureus	ATCC6538	Hazard Group 2
	Staphylococcus aureus	MRSA252	Hazard Group 2
	Staphylococcus epidermis	ATCC14990	Hazard Group 2
	Klebsiella pneumoniae	ATCC13883	Hazard Group 2
2.13 Describe the type and severity of the disease that can be caused to humans, animals or plants by each of the agents and if relevant, the particular strains in use	All HG2 agents can cause disease but treatment is readily available		
2.14 Has any strain listed in Section 2.12 been genetically modified in any way?	○ Yes ⊘ No		

3. CLASSIFICATION OF HAZARD GROUP

3.1. Are you confident that any non-GM organism, tissue, cell, body fluid, excreta or any component thereof covered by this assessment cannot potentially pose a threat to humans or cause human diseases?	○ Yes - Classify as HG1
3.1.1. Can any non-GM organism, tissue, cell, body fluid, excreta or any component thereof cause human disease and potentially be a hazard to humans but is unlikely to spread to the community and for which there is usually effective prophylaxis or treatment available?	⊘ Yes - Classify as HG2
3.1.2. Can any non-GM organism, tissue, cell, body fluid, excreta or any component thereof cause severe human disease and potentially be a serious hazard to humans and that may spread to the community, where effective prophylaxis or treatment may or may not be available?	○ Yes
3.2. Do any of the materials contain pathogens or toxins covered by the Anti-Terrorism Crime and Security Act?	○ Yes ATCSA Schedule 5
ASSIGNMENT OF CONTAINMENT LEVEL	

4. TISSUES, CELLS, BODY FLUIDS OR EXCRETA

4.2. Will any culturing of the material described in section 2 take place? *If Yes, describe which cell(s) will be cultured and under what conditions.*	⊘ Yes ○ No	Keratinocytes and fibroblasts will be cultured under containment level 2 conditions
4.3. Could HIV permissive cells be present*? *If Yes, describe the cells and for how long these cultures will be allowed to grow.* *If unsure seek advice. Refer to CBE Code of Practice for details on additional precautions.*	○ Yes ⊘ No	
4.4. What is the maximum volume of culture grown?	Per Vessel	40 mL
	Number of vessels	50
4.5. Will the tissues, cells, body fluids or excreta be manipulated in any way that could result in the concentration of adventitious biological agent present? *If Yes, explain.*	○ Yes ⊘ No	

4. TISSUES, CELLS, BODY FLUIDS OR EXCRETA

4.6. Will any of the tissues, cells or fluids be donated by you or your colleagues working in or with access to the labs?	○ Yes ⊘ No	

4. BIOLOGICAL AGENTS (ie micro-organisms such as bacteria, viruses, fungi, microscopic endoparasites)

4.8. Describe ALL route(s) of infection (relevant to the laboratory setting) and the minimum infectious dose(s), if known	Name of agent	Route(s)	Minimum infectious dose
	Escherichia coli	Inhalation/Ingestion	
	Staphylococcus aureus	Inhalation/Ingestion	
	Staphylococcus epidermis	Inhalation/Ingestion	
	Klebsiella pneumoniae	Inhalation/Ingestion	

4.9. What is the highest concentration and volume of agent(s) to be worked with?	Per experiment 100mL at 10E8	Total stored 500mL
4.10. Are there any known drug resistances amongst the strains to be used? If Yes, explain what these are and the consequences	⊘ Yes ○ No	S. aureus strain MRSA252 is methicillin resistant. It cannot be treated with methicillin so alternative antibiotics would be needed to treat any infection - but its resistance profile is known and other treatments are available
4.11. What forms of agent will be used e.g. spores, vegetative forms and are there any issues over the robustness of these particular forms e.g. resistance to disinfectants or increased stability on dry surfaces?	Vegetative forms with no known resistance to disinfectant	
4.12. What will be the most hazardous procedure involving the use of this material?	Biofilm culture	

5. RISKS AND CONTROL MEASURES

Risk		How will this be controlled?	Reference to SOP's / Other documentation
5.1. Might infectious droplets, aerosols or splashes be created, either deliberately or by accident?	⊘ Yes ○ No	Some aerosols may be generated during cell culture, manipulation and pipetting of cells. A class II BSC will be used for all cell culture work to protect against aerosols or splashes. All work will be carried out using aseptic technique, maintaining a sterile environment for the cells and also protecting the operator and other users of the laboratory from biological agents using a class II BSC.	SOP038- Biological Spill Response SOP009- Use and maintenance of HERASafre KS Class II BSC
5.2. Will this material be transported within the laboratory e.g. between BSC & incubator?	⊘ Yes ○ No	Sealed filter flasks will be used and be aseptically handled according to SOP005. All microbiological culture will not leave H29.	SOP005-Storage and transport of biological materials
5.3. Will this material (including waste) be transported locally between sites on campus but outside the laboratory?	⊘ Yes ○ No	Not anticipated but any material would be transported in a sealed primary container and a secondary container.	SOP005-Storage and transport of biological materials SOP003- Disposal of biological waste
5.4. Will material(s) listed in section 2.2 or section 2.3 be shipped to organisations elsewhere in the UK or abroad?.	○ Yes ⊘ No		

Risk		How will this be controlled?	Reference to SOP's / Other documentation
5.5. Will this material be received from organisations elsewhere in the UK or abroad?	☑ Yes ○ No	Some bacterial specimens may be received from Dr Rowena Jenkins from Swansea University who has access to clinical diabetic foot ulcer isolates. On receipt, the integrity of the package is checked and then quarantined until it has been deemed suitable for use.	SOP008- Management and Control of Incoming Biological Material
5.6. Will this material be stored?	☑ Yes ○ No	A portion of cells will be cryopreserved in order to maintain a bank of comparable cells to work with. Cryopreservation and thawing of cells will be performed according to the relevant SOPs. When biological agents are in use the separate incubator in H29 will be used. Storage in the fridge will be used where appropriate.	SOP005-Storage and transport of biological materials SOP013- Use and maintenance of liquid nitrogen stores SOP031- Cryopreservation and storage of mammalian cell lines SOP032- Resuscitation of cryopreserved mammalian cell lines
5.7. Will infectious material be centrifuged?	☑ Yes ○ No	Sealed buckets will only be opened in the class II laboratory facility. In the case of a spill, SOP038 will be followed.	SOP038- Biological Spill Response SOP153- Use and maintenance of the H29 centrifuge
5.8. Are biological samples to be cultured in an incubator?	☑ Yes ○ No	Both standard static and shaker incubators at 37C	SOP079- Use and maintenance of Heracell CO2 Incubator SOP053- Use and maintenance of SANYO MCO-18AIC CO2 Incubator
5.9. Are sharps to be used at any stage during this activity?	○ Yes ☑ No		
5.10. Are animals to be used in this project?	○ Yes ☑ No		
5.11. Will a fermenter / bioreactor be used to culture a biological agent or material?	○ Yes ☑ No		
5.12. Is there any stage within the experimental procedures when an infectious material is inactivated (other than for disposal)?	○ Yes ☑ No		
5.13 Are any of the following to be used in conjunction with the project?	☐ Carcinogens or Mutagens		
	☐ Toxins		
You must complete a cryogen risk assessment before work begins and add the reference here.	☑ Liquid Nitrogen	Liquid nitrogen will be in the dewers used for cryostorage	Attached with RA
	☐ Ionising radiation		
You must complete a lone working risk assessment before work begins and add the reference here.	☑ Lone working	Attached with this RA	
5.14. Are there any conditions associated with the hazards described in section 5.13 that require additional control measures?	○ Yes ☑ No		

6. PPE AND HYGENE

Control Measure	Details		Reference to SOPs / other documentation
6.1 When will gloves be worn?	At all times		SOP037- Use of personal protective equipment
6.2 What type and where will they be stored?	Nitrile	In Lab and in Changing Area	
6.3 When will laboratory coats be worn and what type are these?	At all times, except separate green coats to be used in H29	White Howle	
6.4 Where will lab coats be stored and what are the arrangements for cleaning or disposal?	Stored in H32 change room and are regularly sent for cleaning	Green lab coats are stored in H29	
6.5 Provide details of any other types of PPE to be used?	Shoe covers worn at all times, face shields for liquid nitrogen work. Aprons worn over howle coats when necessary. Lab safety glasses when needed.		
6.6 Describe the lab hygiene facilities available and where they are located	Every lab	Designated hand washing facilities are located in each lab	
6.7 Where are the first aid boxes and emergency spill kits located?	First aid boxes are in all labs	Spill kits located in autoclave room, H29, H23	

7. WASTE

7.1 How will waste be treated prior to disposal

(Note that all differently treated wastes must be included e.g. If some liquid is autoclaved, but others not, then describe both)	Treatment prior to disposal	Is the treatment validated?	Reference to SOPs / other documentation
☑ Liquid waste	Treat with Virkon disinfectant prior to disposal. All waste will be labeled appropriately and only processed by the persons involved in the project to ensure correct processing occurs	☑ Yes ○ No	SOP003- Disposal of Biological Waste
☑ Solid waste	Autoclavable decontamination as per SOP003. All waste will be labeled appropriately and only processed by the people involved in the project to ensure correct processing occurs. This includes microbial waste.	☑ Yes ○ No	SOP003- Disposal of Biological Waste SOP024- Use and maintenance of systec VX-95 autoclave CBE044
☐ Other (Specify)			
7.2 Is any waste being autoclaved?		☑ Yes ○ No	SOP024- Use and maintenance of systec VX-95 autoclave CBE044 SOP025- Use and maintenance of systec VX-95 CBE045
All cycles have been validated for the actual load types used? *(If Yes, documentary evidence of the validation must be available)*		☑ Yes ○ No	
The successful completion of every load is checked prior to disposal?		☑ Yes ○ No	SOP024- Use and maintenance of systec VX-95 autoclave CBE044 SOP025- Use and maintenance of systec VX-95 CBE045

7.3 How will liquid waste be disposed of?

☑ To drain?	After 1% Virkon decontamination for 24h	☑ Yes ○ No	SOP003- Disposal of Biological Waste
☐ As solid waste?			

7. WASTE

☐ Other (Specify)			

7.4 How will solid waste be disposed of?

Categorisation	Waste stream colour code	Disposal method (Edit as required)
☐ Sharps		
☐ Sharps contaminated with cytotoxic or cytostatic material		
☐ Human body parts, organs, including blood bags and blood preserves and excreta that have been pretreated before leaving the site		Disinfection or sterilisation in the lab site > Yellow/Orange lidded rigid one way sealed tissue bins > clinical waste disposal (incineration) *Human tissue waste must be placed in separate containers from non-human waste and labelled 'HTA waste'
☐ Animal body carcasses or recognisable parts that have been pretreated before leaving the site		
☑ Potentially or known infected lab wastes contaminated or potentially contaminated with cytotoxic or cytostatic material that have **NOT** been pretreated before leaving the site	**Purple**	Yellow/Purple clinical waste bags > clinical waste disposal (incineration)
☑ Potentially or known infected lab wastes that have **NOT** been pretreated before leaving the site	**Yellow**	Yellow clinical waste bags > clinical waste disposal (incineration)
☑ Infected or potentially infected lab wastes that **HAVE** been pretreated before leaving site	**Orange**	Disinfection or sterilisation in the lab site > orange clinical waste bags > clinical waste disposal (incineration)

8. MAINTENANCE

8.1 Are preventative maintenance and monitoring regimes in place for the following laboratory equipment?

	Inspection / Servicing Frequency	Cleaning / Disinfection Frequency	Monitoring / Alarms Frequency	Reference to SOPs
☑ Centrifuges	Inspected before use and during weekly clean. Serviced after 100-150 hours of use	At the end of each days use and during the weekly clean. Inside the chamber, all parts of the rotation assembly and any head accessories are cleaned and dried	Centrifuge is monitored throughout use	SOP004- General laboratory house keeping
☑ BSCs	Inspected before every use and during weekly clean. Regularly serviced	BSCs are cleaned before and after every use with 1:50 Chemgene and 70% IMS and undergo a deep clean once a week. After each use, BSCs also undergo a round of UV disinfection	Record is kept of downflow velocity (m/s) and performance factor after each use	SOP009- Use and maintenance of HERASAFE KS Class II BSC SOP004- General laboratory house keeping
☐ Fume Hoods				
☑ Autoclaves	Inspected before every use and serviced when needed	Room and autoclaves cleaned weekly. Inside not cleaned as its routinely sterilised during use.	Monitored before use - results from previous run printed off once its complete	SOP024- Use and maintenance of systec VX-95 autoclave CBE044 SOP025- Use and maintenance of systec VX-95 CBE045

8. MAINTENANCE

☑ Incubators	Inspected once a week and regularly by operator prior to use	Incubators are cleaned and decontaminated every fortnight unless a contamination occurs.	Constant monitoring, incubator will sound an alarm if change in temperature or CO2 occurs	SOP079- Use and maintenance of Heracell CO2 Incubator SOP053- Use and maintenance of SANYO MCO-18AIC CO2 incubator
☑ LN2 Stores	Cryobanks checked once a week, delivery of cylinders once a week and stored outside in gas pod	Gas pod - n/a Cryobanks are rotated when LN2 goes cloudy	Gas cylinders are attached to alarms in office	SOP013- Use and maintenance of liquid nitrogen stores
☑ Freezers	Weekly inspection, PAT tested yearly	Cleaned when defrosted as needed	Constant monitoring, connected to alarms in office	SOP016
☑ Fridges	Weekly inspection, PAT tested yearly	Cleaned every month	Constant monitoring, connected to alarms in office	SOP016
Others				
☐ Others				

9. TRAINING

9.1. Have all project research workers undertaken safety training for working with hazardous or potentially hazardous biological materials and agents at CL2?

Name of researcher	Had Training	Date training completed (or will be completed)	If no, state why	+
Jenna Davis	◉ Yes ○ No	November		x

☑ 9.2. This work involves HTA 'Relevant Material', confirm that all project research workers have undertaken HTA training

		Date training completed (or will be completed)				
Name of researcher	Had Training	Induction	On-line	In-house	If No, state why	+
Jenna Davis	⊘ Yes ○ No	4 Sep 2018	4 Sep 2018	23 Oct 2018		x

10. EMERGENCY PROCEDURES

10.1 Are procedures in place for dealing with spillage of infectious or potentially infectious material

Equipment	Reference to SOPs
☑ Within the BSC	SOP038- Biological Spill Response
☑ Within the centrifuge	SOP038- Biological Spill Response
☑ Within the laboratory, but outside any primary control measures (e.g. BSC)	SOP038- Biological Spill Response
☑ Outside the laboratory	SOP038- Biological Spill Response

10. EMERGENCY PROCEDURES

10.2 Describe the procedures in place for an accidental exposure			
Immediate action	Leave the vicinity with anyone present to allow any aerosol to settle for a minimum of 30 minutes. Dispose of any contaminated PPE or outerware and ensure that other users of the area are aware and do not enter until the spill is cleared and it is deemed safe to return	Ref to SOP's	SOP038- Biological Spill Response
When and whom to report the incident	The incident is reported to the lab manager once all staff have exited	Ref to SOPs	SOP038- Biological Spill Response

11. ACCESS

		Explanation	References
11. Is/are the lab(s) adequately separated from other areas (e.g. offices)?	☑ Yes ○ No		
11.2. Is/are the lab(s) or other work areas shared with other users not involved in the project?	☑ Yes ○ No	There is no risk to other lab users. However, to reduce whatever risk may arise, work will be undertaken aseptically in the BSCs as per SOP009, all biological waste will be disposed of as per SOP003 and any used workspace and lab will be cleaned before and after use, as per SOP004. Further to this all microbial work will be performed only in H29 to reduce any potential exposure/ contamination.	SOP009-Use and maintenance of HERASAFE KS Class II BSC SOP003- Disposal of biological waste SOP004-General lab housekeeping
11.3. Describe the measures in place to ensure that hazardous biological agents or material is secure	☑ Yes ○ No	Material will be kept in the laboratory and clearly labeled, with attached biohazard stickers. Liquid reagents will be stored within a secondary container to reduce risk	SOP005- Storage and transport of biological agents

12. OCCUPATIONAL

12.1. All workers involved with handling unscreened blood, blood products and other tissues are recommended to have Hepatitis B immunisation. Have all workers involved in this project been immunized?	☑ Yes ○ No
12.2. Is health surveillance required?	○ Yes ☑ No

13. NOTIFICATIONS

☑ 13.1. Are any of the cells, tissues or fluids covered by the Human Tissue Act (HTA) under the University HTA Licence?	Dermal fibroblasts and keratinocytes
☐ 13.2. Are any of the cells, tissues or fluids obtained from a HTA licensed biobank with REC approval for generic research use?	
☐ 13.3. Does this work have ethical approval from a recognised NHS Research Ethics Committee?	
☐ 13.4. Does any of the work require approval from the University Ethical Committee?	
☐ 13.5. Do any of the materials require approval for use from the UK Stem Cell Bank Steering Committee (MRC)?	
☐ 13.6. Do any of the materials or biological agents listed require any other licenses?	

14. APPROVALS

14. APPROVALS	
Authorised Person	
Departmental Biological Safety Advisor	R I TEMPLE
University Biological Safety Officer (or Deputy)	

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Article

A Novel Three-Dimensional Wound Healing Model

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Abstract: Wound healing is a well-orchestrated process, with various cells and growth factors coming into the wound bed at a specific time to influence the healing. Understanding the wound healing process is essential to generating wound healing products that help with hard-to-heal acute wounds and chronic wounds. The 2D scratch assay whereby a wound is created by scratching a confluent layer of cells on a 2D substrate is well established and used extensively but it has a major limitation—it lacks the complexity of the 3D wound healing environment. Established 3D wound healing models also have many limitations. In this paper, we present a novel 3D wound healing model that closely mimics the skin wound environment to study the cell migration of fibroblasts and keratinocytes. Three major components that exist in the wound environment are introduced in this new model: collagen, fibrin, and human foreskin fibroblasts. The novel 3D model consists of a defect, representing the actual wound, created by using a biopsy punch in a 3D collagen construct. The defect is then filled with collagen or with various solutions of fibrinogen and thrombin that polymerize into a 3D fibrin clot. Fibroblasts are then added on top of the collagen and their migration into the fibrin—or collagen—filled defect is followed for nine days. Our data clearly shows that fibroblasts migrate on both collagen and fibrin defects, though slightly

faster on collagen defects than on fibrin defects. This paper shows the visibility of the model by introducing a defect filled with fibrin in a 3D collagen construct, thus mimicking a wound. Ongoing work examines keratinocyte migration on the defects of a 3D construct, which consists of collagen-containing fibroblasts. The model is also used to determine the effects of various growth factors, delivered in the wound defects, on fibroblasts' and keratinocytes' migration into the defects. Thus this novel 3D wound healing model provides a more complex wound healing assay than existing wound models.

Keywords: fibrin; 3D construct; cell migration

1. Introduction

Wound healing is a complex process that consists of four main stages: hemostasis, inflammation, proliferation, and remodeling of the tissue [6,21]. In the initial stage of wound healing, a fibrin clot is formed at the site of the wound shortly following injury [6,21]. Fibrinogen is cleaved into fibrin monomers by thrombin, and the peptide monomers are polymerized by Factor XIII [21]. The resultant fibrin acts as a scaffold for various cells to move in and out of the wound bed [21]. Five to seven days after the initial injury, fibroblasts migrate to the wound site, secreting new collagen, and keratinocytes migrate from the wound edge and form a thin epithelial cell layer to close the wound [6,21]. Various growth factors secreted by invading cells such as macrophages, fibroblasts, and keratinocytes play an essential role during this process [6,21]. Examples of these growth factors include epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) [1,2,10,14].

In the United States, chronic wounds cost $20–25 billion a year, and acute or traumatic wounds add another $7–10 billion annually. There are highly developed wound products including wound dressings (alginates hydrocolloids and hydrogels), skin substitutes, and growth factor based products that are used to treat chronic wounds [21]. It is known that these products do not work well on all patients, suggesting a lack of understanding of the wound healing process, especially in chronic wounds. To that extent, it is very important to establish a 3D wound healing model that helps with better understanding wound healing in general and cell migration specifically. Existing wound healing models range from simple 2D *in vitro* models to 3D *in vivo* models [3]. In the well-established 2D scratch assay, a wound is created by scratching a confluent layer of cells seeded on a substrate and cell migration into the scratch (wound) is followed [20]. A major limitation of this model is that it lacks the complexity of the wound bed microenvironment. More advanced 3D wound healing models have been established over the last few years. For example, in a wound healing model by Karamichos *et al.*, 2009 [12], fibroblasts are embedded in a 3D collagen construct and the cell migration is followed from the denser collagen matrix into a surrounding matrix. The limitations of this model include the use of only one type of ECM protein and one cell type. An improved 3D *in vitro* model is the "human skin equivalents" [3,22]; it contains a stratified layer of keratinocytes and keeps the surface at an air–liquid interface. In this model, the wound closes in 48–72 h, which is comparable to the *in vivo* environment [3,22]. A third 3D model is an example of a 3D *in vivo* model where skin equivalents are cultured with keratinocytes and then grafted into

mice [9]. Our novel 3D model presented in this study has many advantages including the ability to use more than one substrate and more than one cell type in a 3D construct that mimics the wound bed. This work is based on the extensive work that this lab published over the last 10 years studying the proliferation, migration, and behavior of various cell types including fibroblasts, keratinocytes, monocytes, and mesenchymal stem cells in a 3D fibrin construct [4,7,8,11,15,16,19].

In this paper, we present a novel 3D wound healing model that better resembles the wound bed during the wound healing process. The novelty of this model is the ability to cointroduce multiple cell types and many growth factors and be able to measure the cell migration. This initial paper introduces this new 3D wound healing model. We used fibroblasts as an example to show the complexity and the potential of the model. Ongoing work includes cointroducing fibroblasts in the 3D collagen construct while adding keratinocytes on the top and following the migration of both cells in the defect. We are also using the model to examine the effect of various growth factors introduced in the defect on cell migration. The model could also be used to study the effect of different drugs on cell migration during the wound healing process.

2. Materials and Methods

2.1. Cells

Human foreskin fibroblast (HFFs) lines from ATCC (Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's Medium (DMEM) from CellGro (Manassas, VA, USA) with 4.5 mg/mL glucose, 10% fetal bovine serum, and 5% penicillin/streptomycin. Cells were maintained in an incubator at 37 °C and 5% CO_2. Human epithelial keratinocytes (HEK001, ATCC CRL-2404, Manassas, VA, USA) were cultured in keratinocyte serum-free media (GIBCO) in 5% CO_2 at 37 °C. The medium was changed every three days, and cells were passaged to new flasks upon reaching confluence. Passage 6–10 keratinocytes were used.

2.2. 2D Scratch Wound Assay

One milliliter of protein-containing solution (fibrinogen or collagen) was pipetted into a 12-well plate. The well plate (polysterene) was used as a comparative control. After a one-hour incubation to allow proteins to adsorb to the surface, the remaining solution was removed and the surface was washed twice with DPBS. Eighty thousand cells/cm^2 were seeded in each well. A reference point was drawn on the bottom of each well. The plate was incubated overnight to allow the cells to adhere to the substrate.

The next day, a pipette tip was used to scratch the confluent cell layer. A Leica DM IRB bright-field microscope was used to image each scratch wound at the reference point. This process was repeated at hourly time points to monitor the cell migration to close the wound area.

2.3. Preparation of the 3D Model

Biopsy Punch Fabrication

Seven hundred microliter 2.5 mg/mL Purecol (Advanced Biomatrix, San Diego, CA, USA) collagen gels were formed in a 12-well plate. NaOH (0.1 N) was added to the collagen and 1 × PBS to neutralize and polymerize the collagen. The well plate was placed in the incubator at 37 °C for an hour to allow the collagen to fully polymerize. HFFs were trypsinized using TrypLE Select (Gibco). A specific number of HFFs were isolated to give a final concentration of 3×10^5 cells/gel and stained with Vybrant DiO (Invitrogen, Carlsbad, CA, USA). After staining, the cells were washed three times, then seeded onto the collagen gel. After 3 h, a 2 mm biopsy punch attached to a vacuum line was used to punch regions out of the collagen gel. These defects were then filled with 2.5 mg/mL collagen or 10 mg/mL purified fibrinogen (Enzyme Research, South Bend, IN, USA) with 10 IU/mL thrombin (Gibco, Carlsabad, CA, USA). Once the defects were polymerized, 1 mL of media was added to each sample well.

2.4. Preparing Conditioned Media

For each 3D model, a 2D layer of confluent HFFs was seeded in a separate well plate. We prepared the conditioned media in a separate well plate; the number of wells needed corresponded to the total sample wells containing the 3D model: For the scratch-conditioned media, fresh medium was added, and the 2D layer of cells was scratched with a pipette tip 24 h prior to the time point. For the confluent conditioned media, fresh medium was supplied to a confluent 2D layer of HFFs 24 h prior to the time point. After each time point, the medium from the 2D layer was removed, spun down, and added to the 3D construct. For the control, fresh medium was added.

2.5. Measuring Cell Migration

Prior to imaging, the gel surface was gently rinsed three times with medium. The construct was imaged using a Nikon Eclipse Ti fluorescent microscope at day 0, 3, 6, and 9. The FITC filter was used to image the HFFs. The images were further analyzed using ImageJ software to quantify the distance between migrated cells and the wound edge. Day 0 was treated as our reference, since the defect area shrunk as HFFs moved toward the center of the defect, and the migration rate was obtained by plotting the data points from ImageJ.

2.6. Statistical Analysis

Data were presented as the mean ± standard error, with n = 3 (triplicate constructs per condition, repeated three times). Data were assessed using Student's *t*-test, with $p < 0.05$ considered significant.

3. Results

3.1. Cell Migration in 2D Scratch Assays

In this experiment, we created a scratch in a confluent layer of fibroblasts seeded on collagen, fibrin, or polystyrene and measured the fibroblasts migration over two days. Visual analysis (Figure 2) showed