Antimicrobial Functionalization of Ovine Forestomach Matrix with Ionic Silver

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Background & Objective

The wound environment is characteristically contaminated with microorganisms. Should contamination progress unchecked to critical colonization or infection, this may adversely affect wound healing outcomes, patient safety and the economy of care. Dressings with antimicrobial functionality to prevent microbial colonization of the dressing are a useful tool to mitigate such complications of microbially at-risk wounds.

The decellularized extracellular matrix biomaterial, Ovine Forestomach Matrix* (OFM), is an established scaffold for use in wound management and tissue repair indications. An OFM variant incorporating ionic silver, termed OFM-Ag, has recently been developed as a wound dressing to combine the benefits of an extracellular matrix scaffold with antimicrobial functionality.

This study sought to characterize the functional properties of OFM-Ag in relation to its intended use as an antimicrobial wound dressing. The antimicrobial effectiveness spectrum and wear time of the dressing were assessed, in addition to dressing silver concentration, kinetics of silver release and dressing cytotoxicity. The structural integrity of the extracellular matrix scaffold was also assessed to rule out any structural damage to the collagen scaffold as a result of incorporating ionic silver.

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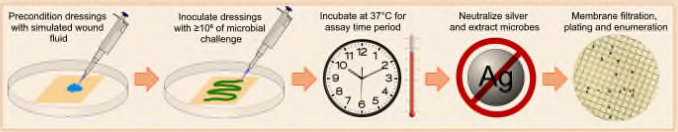
Methods

Antimicrobial Effectiveness

The ISO 20743 absorption method including ASTM E1054 validated neutralization procedure was used to assess OFM-Ag dressing antimicrobial effectiveness. Antimicrobial spectrum determination utilized a panel of clinically relevant wound colonizing species (including drug resistant strains) with antimicrobial wear time assessed at 1, 3 and 7 days after microbial challenge and incubation under physiological conditions.



Silver Quantification and Elution Kinetics

Silver quantification was performed by atomic absorption spectrometry (AAS). Dressings were hydrolyzed in concentrated nitric acid and digests analyzed via air-acetylene flame AAS. Silver elution kinetics were characterized via elution using pure water (0.29 mL/cm²/day) at 37 °C over a 7 day time course, with water removed and replaced daily. At the 1, 3 and 7 day time points dressings were removed from elution, lyophilized, hydrolyzed with nitric acid and silver quantified by AAS.

Cytotoxicity

Cytotoxicity testing was performed via the ISO 10993-5 MTT method using murine fibroblasts (3T3 cell line). A commercial collagen/ORC-silver dressing was also included. Triplicate assays were performed, where dressings were hydrated in saline (per respective IFUs) and extracted in cell culture media according to ISO 10993-12. Undiluted extracts were applied to cell monolayers (n=6 per dressing, per assay) and incubated for 24 hours. MTT solution was added and cell viability quantified via MTT reduction measured by optical density at 570 nm.

Collagen Structure

Integrity of dressing collagen structure was assessed using differential scanning calorimetry (DSC) and scanning electron microscopy (SEM). For comparative purposes, DSC/SEM analysis included a commercial collagen/ORC-silver wound dressing and DSC included OFM and unprocessed ovine forestomach tissue (OFT). For DSC, samples were hydrated in phosphate buffered saline (5 minutes) and 5-20 mg hermetically sealed in aluminum crucibles. The temperature was equilibrated (10°C) before ramping to 120°C (8°C/min). Sigmoidal peak integration was used to calculate onset melt temperature. For SEM, 2x5 mm samples were mounted on aluminum stubs and viewed using a Hitachi TM3030 (University of Auckland, New Zealand) with 15 kV accelerating voltage.

Results & Discussion

Antimicrobial Effectiveness Spectrum & Wear Time

OFM-Ag dressings demonstrated a high degree of antimicrobial effectiveness across a spectrum of 11 microbial species of relevance to wound care including representatives of gram positive bacteria, gram negative bacteria, yeast and mold. The sustained effectiveness of OFM-Ag over a 7-day wear time period indicates prolonged antimicrobial protection of the dressing, rather than a transient initial knockdown.

Antimicrobial Effectiveness of OFM-Ag Dressings

	Microbial Species	Log_{10} Reduction 1 Day	3 Days	7 Days
Gram positive bacteria	Methicillin Resistant *Staphylococcus aureus* (MRSA)	7.0	>8.5	7.8
	Staphylococcus epidermidis (coagulase negative)	8.3	>8.6	>8.6
	Streptococcus pyogenes (Group A, β-hemolytic)	>7.6	>7.6	>7.6
	Vancomycin Resistant *Enterococcus faecalis* (VRE)	7.5	7.8	>8.2
Gram negative bacteria	*Pseudomonas aeruginosa*	>9.6	>9.6	>9.6
	Escherichia coli	>6.9	>6.9	>6.9
	Acinetobacter baumannii	>8.6	>8.6	>8.6
Yeast & mold	*Aspergillus brasiliensis (niger)*†	1.8*	>5.3	>5.3
	Candida albicans	6.1	>8.9	>8.9
	Candida glabrata	6.6	>8.5	>8.5
	Candida parapsilosis	7.3	>7.6	>7.6

> prefix indicates greater-than as no viable microbes remained on any test replicates (maximum log reduction acheivable for the assay or "complete kill").
†*A. brasiliensis* testing utilized a spore suspension inoculum for standardization/enumeration purposes.
**A. brasiliensis* 1 day result is an artifact of the spore inoculum used, spores remained dormant on dressing (unable to grow/colonize the dressing) but recovered following neutralizing extraction and plating to nutrient rich media.

Silver Quantification & Elution Kinetics

The silver concentration of OFM-Ag dressings was determined to be 0.30% (w/w) or 12 µg/cm².

OFM-Ag Dressing Silver Elution Profile and Elution-Antimicrobial Effectiveness

Dressing Elution Time	Log_{10} Reduction vs *S. epidermidis*
Day 0 (pre-elution)	>9.0
Day 1	>8.9
Day 3	5.0
Day 7	6.5

Antimicrobial effectiveness testing of eluted dressings utilized 1 day assay incubation period; > prefix indicates greater-than as no viable microbes remained on any test replicates (maximum log reduction achievable for the assay or "complete kill"). Error bars represent standard deviation of n=6 replicate dressings per time point.

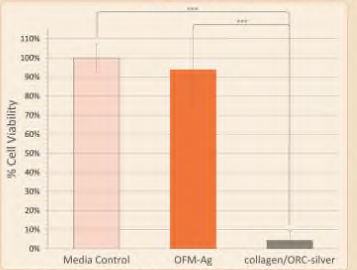
Under maximal elution conditions or "worst case scenario" of elution, the elution profile of silver from OFM-Ag dressings was gradual but sustained over the first 3 days of elution, after which no substantial amount of silver was released. After 7 days of elution, dressings had lost only ~40% of the initial silver content.

Antimicrobial effectiveness testing of eluted OFM-Ag dressings toward the gram positive bacterium *S. epidermidis* demonstrated that OFM-Ag retained effective antimicrobial protection throughout the 7 day period of maximum silver elution.

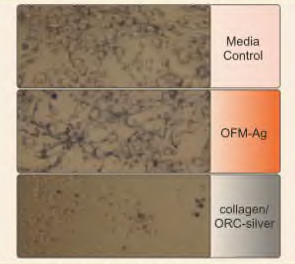
Cytotoxicity

Silver is a well known antimicrobial, however depending on concentration, form and presentation silver may impart cytotoxic effects detrimental to wound healing. Therefore it is vital to balance the antimicrobial effects of silver while maintaining dressing biocompatibility.

Response of Mammalian Cells to Silver Dressings



Error bars represent standard deviation (n=18 replicates from n=3 experiments).
*** indicates statistically significant difference between groups (p<0.01)



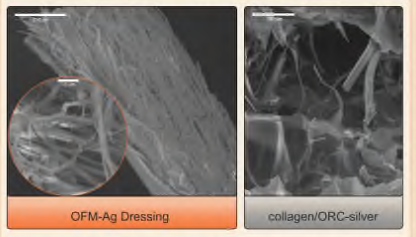
Representative micrographs (40x objective) of cell layers post-MTT. Purple coloration is MTT reduced to formazan by viable cells.

OFM-Ag dressings were well tolerated by mammalian cells, exhibiting no statistically significant difference in cell viability relative to the media control (p>0.05). In contrast, collagen/ORC-silver dressings elicited a marked cytotoxic response. As measured in these standard MTT viability experiments, OFM-Ag dressings were ~80% less cytotoxic compared to collagen/ORC-silver.

Collagen Structure

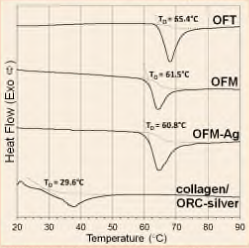
The intact collagen structure of extracellular matrices is known to confer benefits to wound healing. Collagen matrix integrity was measured by melt onset temperature (T_O) via DSC. For reference, healthy human dermis has a melt onset of ~60°C, whereas degraded collagen can be identified by decreased melt onset temperature.

SEM of Silver Wound Dressings



SEM images taken at 500x magnification, OFM-Ag image insert 3000x magnification.

Silver Wound Dressing DSC



Onset melt (T_O) values are mean of n≥22 replicates.
Y-axis scale is arbitrary to allow overlay of thermograms.

There was no significant difference (p>0.05) in T_O between OFM and OFM-Ag, demonstrating the inclusion of ionic silver does not damage the collagen matrix. The T_O of collagen/ORC-silver was notably lower, indicative of degraded collagen (i.e. gelatin) without native matrix structure.

SEM imaging concurred with DSC results, showing collagen/ORC-silver to have a very porous structure threaded with cellulosic strands but no fibrillar collagen present. OFM-Ag exhibited an intricate architecture of interwoven heterogeneous collagen fibrils characteristic of a native extracellular matrix.

Conclusions

Characterization studies have determined the following properties of OFM-Ag:

- Broad spectrum antimicrobial effectiveness over a 7 day wear time.
- Contains 0.30% (w/w) silver, equivalent to 12 µg/cm².
- Maintains antimicrobial effectiveness over 7 days of elution.
- Well tolerated by mammalian cells, exhibiting no cytotoxic response.
- Retains the native extracellular matrix structure of OFM.

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