Poly(ethylene glycol)-Based Hyperbranched Polymer from RAFT and Its Application as a Silver-Sulfadiazine-Loaded Antibacterial Hydrogel in Wound Care

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Poly (ethylene glycol) based hyper-branched polymer from RAFT and its application as a silver sulfadiazine loaded anti-bacterial hydrogel in wound care.

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Keywords: hydrogels, thiol-ene click chemistry, poly(ethylene glycol), hyperbranched polymer, hyaluronic acid, antibacterial, wound care

Abstract: A multifunctional branched co-polymer was synthesized by Reversible Addition-Fragmentation Chain Transfer polymerisation (RAFT) of poly (ethylene glycol) diacrylate (PEGDA M_n=575) and poly (ethylene glycol) methyl methacrylate (PEGMEMA M_n=500) at 50:50 molar ratio. Proton nuclear magnetic resonance spectroscopy (^{1}H NMR) confirmed a hyper branched molecular structure and a high degree of vinyl functionality. An *in situ* cross-linkable hydrogel system was created via a "click" thiol-ene type Michael addition reaction of vinyl functional groups from this copolymer with thiol-modified hyaluronic acid, a natural immunoneutral polysaccharide. Further encapsulation with antimicrobial silver sulfadiazine (SSD) was conducted to create an advanced antimicrobial wound care dressing. This hydrogel demonstrated a sustained antibacterial activity against the bacteria *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* at a moderate level comparing to the direct topical application of SSD. In addition, in vitro toxicology evaluations demonstrated that this hydrogel, with low concentrations of SSD supported the survival of embedded human adipose derived

stem cells (hADSC's) and inhibited growth of mentioned pathogens. This study demonstrates that this hydrogel encapsulated with a low concentration (1.0% w/v) of SSD can be used as a cell laden gel system with the ability to inhibit growth of pathogens without posing an unacceptable threat towards ADSC's.

1. Introduction

In our quest to develop an advanced wound care dressing, hydrogels with multiple functions were investigated. Highly branched copolymers have been studied over the past decade while our research focus is on their applications in medicine and the wound care market including hydrogels. Pathogens are proving more difficult to eradicate as bacteria continue to develop worrying resistance towards antibiotics. This bacterial resistance is growing daily in both community and hospital settings and is leading to increased mortality and morbidity[1]. For this reason, new innovative approaches, including reinvigoration of tried and tested agents with novel delivery must be investigated to prevent infection and encourage wound repair.

Hydrogel technology is a rapidly advancing field with a wide range of applications[2 3 4] . Hydrogels are composed of a high percentage of water which provides a perfect moist environment for tissue regeneration[5] while also preventing contraction of the wound. Additionally, these hydrogel systems can be engineered to deliver cells and drugs[6]. This proposed moist environment can provide a rich supply of white blood cells, enzymes, cytokines and growth factors[7]. Primarily released enzymes from white blood cells can cause selective autolytic debridement of necrotic tissue[8]. Unfortunately this moist environment can also provide an ideal site for the colonization of bacteria [9]and therefore hydrogels should be applied with a synergistic antimicrobial agent[10].

It is clear that the natural defence systems of organisms are highly advanced; nonetheless, the regeneration process is often delayed and hampered by a number of factors[11]. The forefronts include infection and an inappropriate wound environment. Concerns in the treatment of wounds, and indeed

many other pathogenic conditions currently rest with the ever threatening development of bacterial resistance[12]. Worldwide, bacterial resistance has increased dramatically over the past few years[13] and is recognised as a highly significant medical challenge. Many bacterial species are now resistant to antibiotics and some Gram-negative bacteria, especially the *Pseudomonas aeruginosa* species have developed resistance to most or all available antibiotics[14]. Furthermore, since the year 2000 only three new classes of antibiotics have been introduced to the market for human use[15]. Despite the certain need for novel antibiotics without cross-resistance issues, research and development has not yet delivered[16], thus failing to provide an answer to the fast emergence and spread of these dangerous bacteria. It is the focus of this work to develop an approach that is bio-medically engineered with strategic design to counteract the fast development of bacterial resistance that in the past has rendered many treatments obsolete. Novel treatments are clearly slow to develop and achieve regulatory approval; thus it has been the aim of this research to re-invigorate use of an age-old antimicrobial agent, such as silver sulfadiazine (SSD), in medical wound care dressings/delivery for a lasting more effective antibacterial dressing with improved stimulation of wound regeneration in combination with cell therapy. Proof of concept research finding with this antibacterial agent will demonstrate the potential for this system being used in combination with many other antibacterial agents.

A recent review into the mechanisms of bacterial resistance has detailed three important cases including *E. coli* resistance to third-generation cephalosporins, the emergence of vancomycin-resistant *S. aureus*, and multidrug resistance in *P. aeruginosa*[17]. Both *S. aureus* and *P. aeruginosa* are recognised as ESKAPE pathogens emphasizing their capacity *to "escape"* from common antibacterial treatments[18]. Research suggests that these bacteria are developing resistance through a host of mechanisms[19]. Aside from bacteria being intrinsically resistant to ≥1 class of agents, they may also acquire their resistance by mutation or acquisition of resistance genes from other organisms. These can enable a bacterium to produce an enzyme to supress the antibacterial agent, to prevent an agent reaching its target, to change its target site or to produce an alternative metabolic pathway to bypass the action of the drug entirely[20]. This

suggests that a long term effective treatment will need to employ a range of inhibitory mechanisms. This will effectively prolong the development of resistance by ensuring that development of one resisting trait will not be sufficient to prevent bacteria death.

The anti-bacterial effectiveness of silver has a long history. It has been used for at least six millennia to prevent microbial infections. The first known record of silver nitrate being used as a medical agent was reported by Gabor in the year 702 and in the year 980 A.D. as a blood purifier and to treat palpitations of the heart[21]. By the 1800s it was taken as common practice (for wealthy) to store wine, water and milk in silver containers to keep them fresh for longer. Nano-Ag particles and their mechanisms of inhibition are a topic of disputed interest but have demonstrated the ability to destabilize the outer membrane, collapse the plasma membrane potential and deplete the levels of intracellular ATP of *E. coli*[22]. These silver based agents are particularly effective against a wide range of pathogens[23] and it remains particularly difficult for bacteria to develop resistance to the action of silver due to the range of inhibitory mechanisms evoked[24]. It is well known that silver ions and silver based compounds are highly effective against micro-organisms exhibiting potent antibacterial activity on as many as 16 species of bacteria[25].

SSD is a similar drug to silver nano particles in its mechanism of inhibition and is typically delivered in a 1% cream or aqueous suspension. It has proved extremely effective for burn wounds to the extent that it is the current gold standard treatment used to treat serious burn wounds but presents opportunity for cross application in other high infection risk areas. This is a highly efficient inhibitor of microbial growth belonging to a family of drugs called sulfa antibiotics. Silver is complexed to propylene glycol, steryl alcohol and isopropyl myrislate. This is mixed with the antibiotic Sulfadiazine to achieve a combined formulation that is highly effective. By substituting a silver atom for a hydrogen atom in the sulfadiazine molecule it results in a combination of the inhibitory action of the silver with the anti-bacterial effect of the sulfadiazine[26 27]. This antibacterial agent is appropriate for hydrogel incorporation in its powder form. It is relatively inexpensive, easy to apply, well tolerated by host cells and has good activity against most pathogens but more importantly it employs a range of mechanisms which makes it difficult to develop

resistance against[28] [29]. Interest in these silver based antibacterial agents has recently been rejuvenated, mainly due to the spread of methycillin-resistant *S. aureus* (MRSA) and the associated reduction in effective antibiotics. This silver sulfadiazine combination is highly effective which can be explained by the strong bonding that occurs between this silver compound with DNA which differs from silver salts alone. Furthermore this silver sulfadiazine combination has an increased effectiveness on disrupting the bacterial wall. Therefore, SSD has been selected for this application due to the broad spectrum of activity and the significantly lower propensity to induce microbial resistance than other antibiotics[30]. However, a high dosage of SSD through topical administration could be toxic and cause side effects.

We hypothesise that the encapsulation of SSD within a PEG based 3D hydrogel scaffold can provide an enhanced wound regeneration environment with controlled release of an antimicrobial agent and ease the need for improved anti-resistant solutions. This advanced antimicrobial hydrogel can be used for delivery of therapeutic cells while preventing cell death caused by infection. In this work, a hydrogel was created by combining our *in situ* crosslinkable copolymer with thiol modified (SH) hyaluronic acid (HA). Hyaluronan or hyaluronic acid (HA) is a linear d-glucuronic acid and N-acetyl-D-glucosamine copolymer[32] and is known as a good biopolymer for many biomedical applications[33]. This report presents experimental data on the synthesis of hydrogels, loading of SSD, antibacterial disk diffusion tests and cell viability assays, building on a previous publication[34].

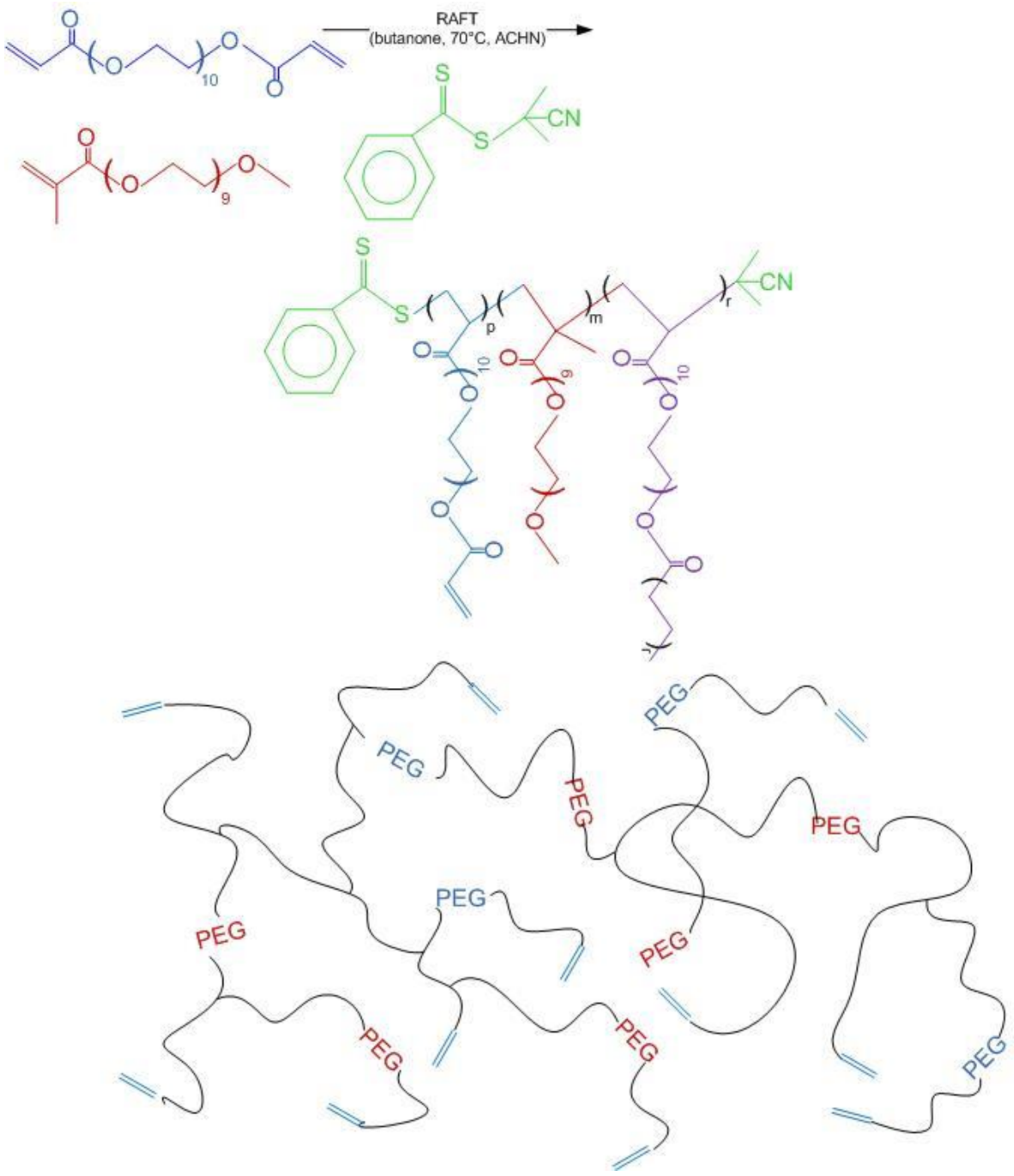

Figure 1. Preparation of highly branched polymer from the monomers PEGDA and PEGMEMA using the RAFT polymerisation technique initiated by ACHN. The highly branched polymer structure contains multiple vinyl functional groups, which will react with thiol functional groups in HA-SH via thio-ene addition reaction mechanism, to form cross-linked hydrogels.

2. Materials and Methods

2.1. Materials

Silver Sulfadiazine (SSD) Powder (98%), Poly (ethylene glycol) methyl ether methacrylate (PEGMEMA, M_n= 500 g mol^{-1}), Poly (ethylene glycol) diacrylate (PEGDA, M_n = 575 g mol^{-1}) and the initiator: 1, 1′-Azobis cyclohexanecarbonitrile (ACHN) were purchased from Sigma-Aldrich. The RAFT agent was synthesised according to published method[34]. Thiol-modified hyaluronic acid (HA-SH) was purchased from Glycosan. Bacterial strains *S. aureus, P. aeruginosa* and *E. coli* were supplied by the Microbiology group at NUI Galway.

2.2. Methods

2.2.1. Synthesis of PEGDA-PEGMEMA Hyperbranched Copolymer

The multivinyl hyperbranched PEGDA-PEGMEMA co-polymer was synthesized via RAFT polymerisation from the monomers poly (ethylene glycol) diacrylate (PEGDA M_n=575) and poly (ethylene glycol) methyl methacrylate (PEGMEMA M_n=500) at 50:50 molar ratio according to previous published method[34] (**Figure 1**). PEGDA-PEGMEMA co-polymer was analysed using ^{1}H NMR to confirm its structure and composition and using GPC to obtain its M_w and PDI. The data can be found in the supporting information (SI).

2.2.2 Preparation of Hydrogel Samples

A 10% PEGDA-PEGMEMA copolymer solution was prepared using 1X PBS solution. 150µL of this polymer solution was transferred into separate eppendorfs under sterile conditions. SSD was measured by weight in its powder form and added to these eppendorfs to create concentrations of 0.0, 0.1, 1.0 and 5.0% (see Table S4 in supplementary information). A volume of 150µL of thiol modified Hyaluronan was added to each Eppendorf creating a 1:1 copolymer: hyaluronan ratio in order to cross link and create hydrogels as shown in **Figure 2**. The addition of Hyaluronan was conducted to one eppendorf at a time, followed by rapid mixing and pipetting of 50µL samples to a clean sterile teflon surface under a flow hood. The samples formed globular shaped gels due to rapid cross linking within 2 minutes. This process

was repeated to create sufficient hydrogel (50µL) samples to conduct experiments in triplicate (n=3) for all SSD concentrations against three bacterial strains. These samples contained the SSD at concentrations of 0.0, 0.1, 1.0 and 5.0% w/v.

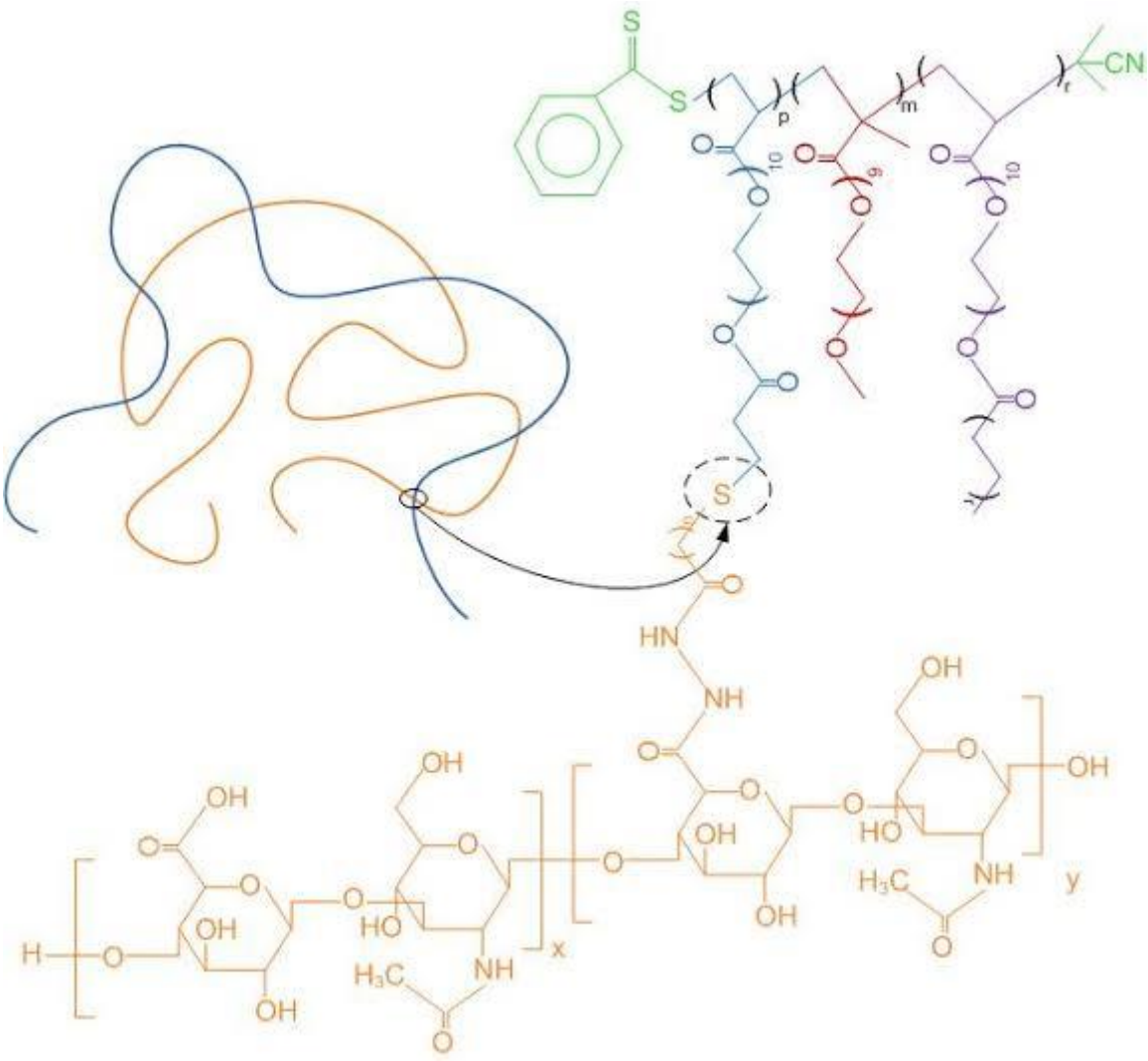

Figure 2. Hydrogel sample preparation by cross linking of the vinyl groups on the polymer chains using a thiol modified hyaluronic acid. Polymer and thiol modified hyaluronic acid were represented by blue and tan interconnected lines while chemical structure at the cross link site was illustrated. (Tan structure represents HA-SH).

2.2.2. Antibacterial Activity

S. aureus, P. aeruginosa and *E. coli* were used as test organisms given their relevance as infectious agents and affinity to develop bacterial resistance. Bacteria were prepared and cultured in fresh Mueller Hinton Agar (MH). One loopful of bacteria was inoculated in a test tube of 5mL Luria Broth (LB) and grown, with shaking, at 37°C for 24 h. The optical density of these solutions was then measured using a Thermo Fisher Scientific Biomate 3 Spectrometer. These cultures were diluted in LB broth to an OD_{600} of 0.8 and moved to an incubator at 37°C with shaking at 250 RPM to re-grow bacteria to an OD_{600} of 0.1.

A standard disk diffusion test was then performed in accordance to the protocol outlined in the Kirby-Bauer test[35]. Mueller Hinton agar plates of 20 ml volume were prepared and 100µL samples of bacterial cultures were spread on plates. After 30 minutes incubation at room temperature, hydrogel samples containing varied SSD concentrations (0.0, 0.1, 1.0 and 5.0%) were added in triplicate to produce a total of 12 plates containing 36 hydrogel samples (3 bacterial strains x 4 SSD concentrations and n=3). All plates were incubated for 72 hours at 37°C, with zones of inhibition measured after 24, 48 and 72 hours using a Vernier Calliper's. Measurement of zones was conducted by measuring three randomly orientated diameters from each hydrogel sample. Note, hydrogels containing SSD < 0.1% were not studied for antimicrobial activity as the literature suggests[36] that concentrations below this nominal value are not as effective in maintaining antimicrobial environments.

2.2.3. Diffusion Studies

A simple release assay[37] was conducted to investigate the release of these SSD particles over an extended period of time in PBS buffer. Initially the optimum wavelength for detection of particles was identified using spectroscopy (290nm) for assessing the presence of SSD particles. After identification of the appropriate wavelength, release studies were conducted on hydrogel samples containing SSD which were

prepared in triplicate (n=3) as previously described at the concentrations of 0.0, 0.1, 1.0 and 5% w/v. The release assay was conducted over 168 hours, time points taken at 2, 4, 21, 51 and 168 hours.

2.2.4. Cytotoxicity Testing

2.2.4.1. Cell Culture and Cell Count

Adipose derived stem cells (ADSC's) were passaged in a $1.75cm^3$ culture flask and incubated at 37°C and 5% CO_2 with media changed every 2-3 days. The media used was Dulbecco's Modified Eagles medium with Bovine Fetal 10% bovine serum and 1% penicillin streptomycin.

Working in conjunction with standard protocol[38] a cell count was conducted. Taking a 96-well plate, 20uL of Trypsin Blue was added to 3 of the wells. To the first well, 20uL of cell solution was added resulting in a 50% dilution. This was then diluted to 25% and 12.5% solutions by adding 20uL from the 50% and 25% wells. The study determined a cell density of $2.8\ x\ 10^5$ cells/ml.

2.2.4.2. Cell Viability

Cell viability was evaluated using the AlamarBlue assay following the protocol[39] using freshly prepared (sterile) SSD encapsulated hydrogels. Experiments were conducted in triplicate to assure confidence. Hydrogels containing concentrations of 0.0, 0.005, 0.01 and 1.0% of SSD were synthesized for the experiment as explained previously. A concentration of 5.0% SSD was not tested as the literature reported that this level is too high for good cell viability[40].

Briefly the experiment was prepared by adding 50,000 cells and 2ml DMEM to each well (Total of 12 wells per plate and labelled accordingly). In triplicate these gels were added to wells. Plates were all incubated at 37°C and 5% CO_2. On days 1, 3 and 7 cell viability was assessed using AlamarBlue assay.

3. Results

3.1. Preparation of Hydrogel Samples

The hydrogel samples were prepared using a PEGDA-PEGMEMA hyperbranched copolymer with the composition of of 40:60 and molecular weight (M_n) of 15.5 KDa, which was synthesized using the feed molar ratio of PEGDA and PEGMEMA as 50:50 according to previously published methods (see SI for more information) This copolymer was crosslinked with thiol modified hyaluronic acid via thiol-ene click chemistry and loaded with SSD to create a matrix represented by **Figure 3**. These hydrogels (50µL) were prepared on sterile Teflon, which were uniform, soft and semi-transparent depending on the concentration of SSD used. Hydrogels exhibited a whitish colour with increased addition of SSD. All samples were easily removed from the Teflon tape and were globular in shape as shown in Figure 4. Hydrogels were structurally stable and kept their shape throughout process presenting little difficult in handling. Samples could be inverted on Teflon slide without detachment or shape changes, exhibiting good structural conformity and well defined cross linking network.

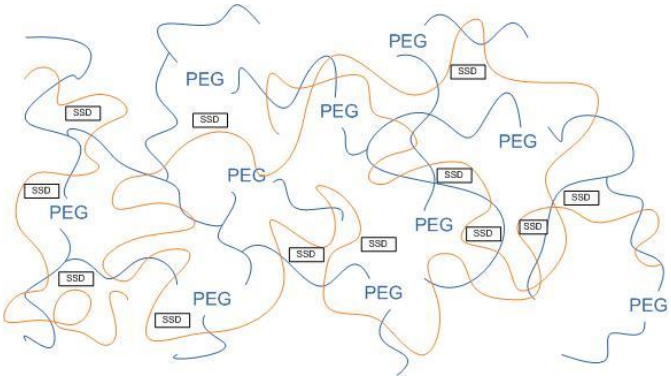

Figure 3. SSD loaded crosslinked hydrogels. Prior to the addition of HA-SH to create crosslinked gels with PEG based hyperbranched copolymer, the copolymer was dissolved in water and mixed with SSD as an aqueous solution.

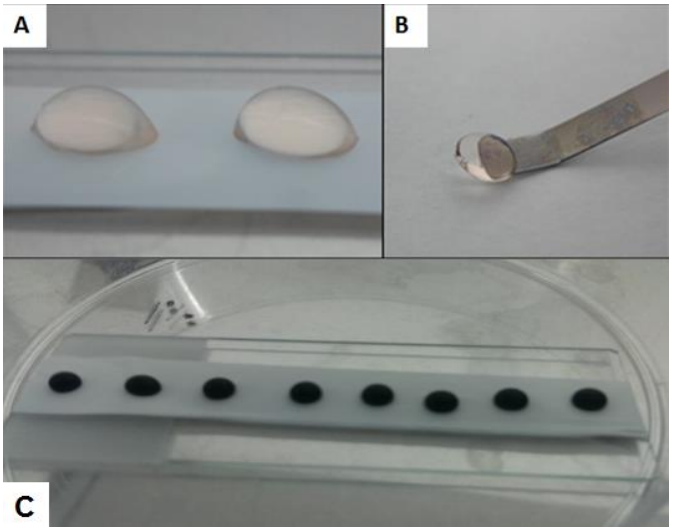

Figure 4. (A) Hydrogel samples (40 - 50µl) with no loading and (B) Hydrogel (without loading) illustrating good workability and ease of use (no deformation in handling). (C) Hydrogel samples loaded with silver nano particles (SNP) (Black). Note, SNP loaded gels shown in image (C) as the black colour presents clear contrast. Silver sulfadiazine (SSD) encapsulated hydrogels present as white hydrogels.

3.2. Antibacterial Activity

The zone of inhibition of bacteria was dependant on the diffusion of antibacterial agent as well as the SSD % presence. The zones of inhibition were clearly visible and are shown in Figure 5. When compared with silver nano particle (SNP's) loading in the same concentration, the SSD samples proved more effective as inhibiting agents for combination with this particular hydrogel, some SNP loading and results are presented in supplementary information. These SSD loaded hydrogel samples displayed large zones of inhibition after a period of 24 h. These zones were inspected again at 48h and 72 h. The zones of inhibition did not change notably after these periods of time. Areas outside of the SSD diffusion showed high bacterial development notable by colour contrast in Figure 5. Colour images of plated bacteria are presented in the supplementary information, notice the light amber colour of (A) *E. coli*, the green of (B) *P. aeruginosa*, and the tan colour of (C) *S. aureus* in Figure S11.

Zones of inhibition are present in all SSD hydrogel samples with best results at 1% and 5% w/v concentrations. No zones of inhibition are present in control samples indicating the presence of SSD is required to prevent bacterial growth. This also demonstrates that the hydrogel allows diffusion of SSD and that SSD inhibits growth of each strain of bacteria tested with inhibition zones dependent on the diffusion behaviour.

The study was repeated at increased concentrations of SSD, from 0.0%, 0.1 %, 1% to 5% in hydrogel samples. Test data from 1% and 5.0% proved to be equally efficient for inhibiting bacteria growth in terms of diffusion zones with no significant difference being observed. This suggests that a concentration of 1% SSD may be optimal for this particular hydrogel; however, diffusion limitations could be a contributing factor to inhibition zones. These results demonstrate that the use of SSD as an antibacterial agent is highly suitable in this hydrogel system since relatively low concentrations of 1% w/v SSD can readily diffuse in an acceptable timeframe. Inhibition of bacterial growth from 3 bacterial strains, including both Gram-positive and Gram-negative species, demonstrates good potential for this hydrogel to be used across a variety of bacterial pathogens.

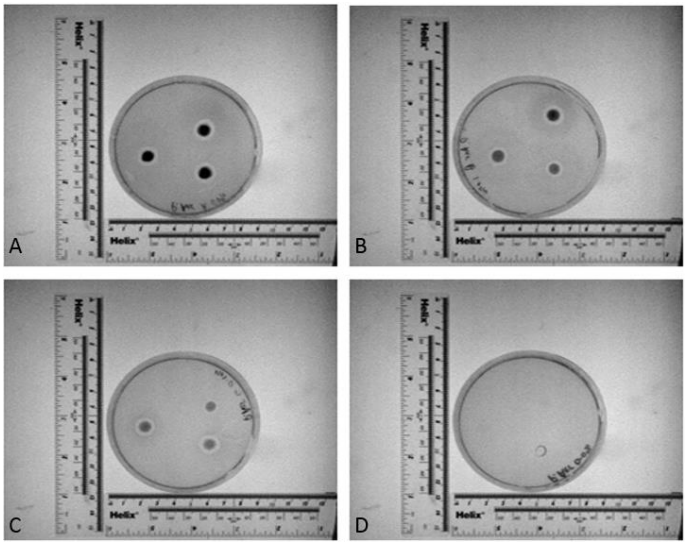

Figure 5. *P. aeruginosa* plated in agar containing uniform hydrogel samples loaded with silver sulfadiazine (SSD). (A) 5.0% w/v, (B) 1.0% w/v, (C) 0.1% w/v, (D) 0.0% w/v. Note, hydrogel contrast is

more visible at higher SSD concentrations and gel samples without SSD appeared transparent as observed in (D).

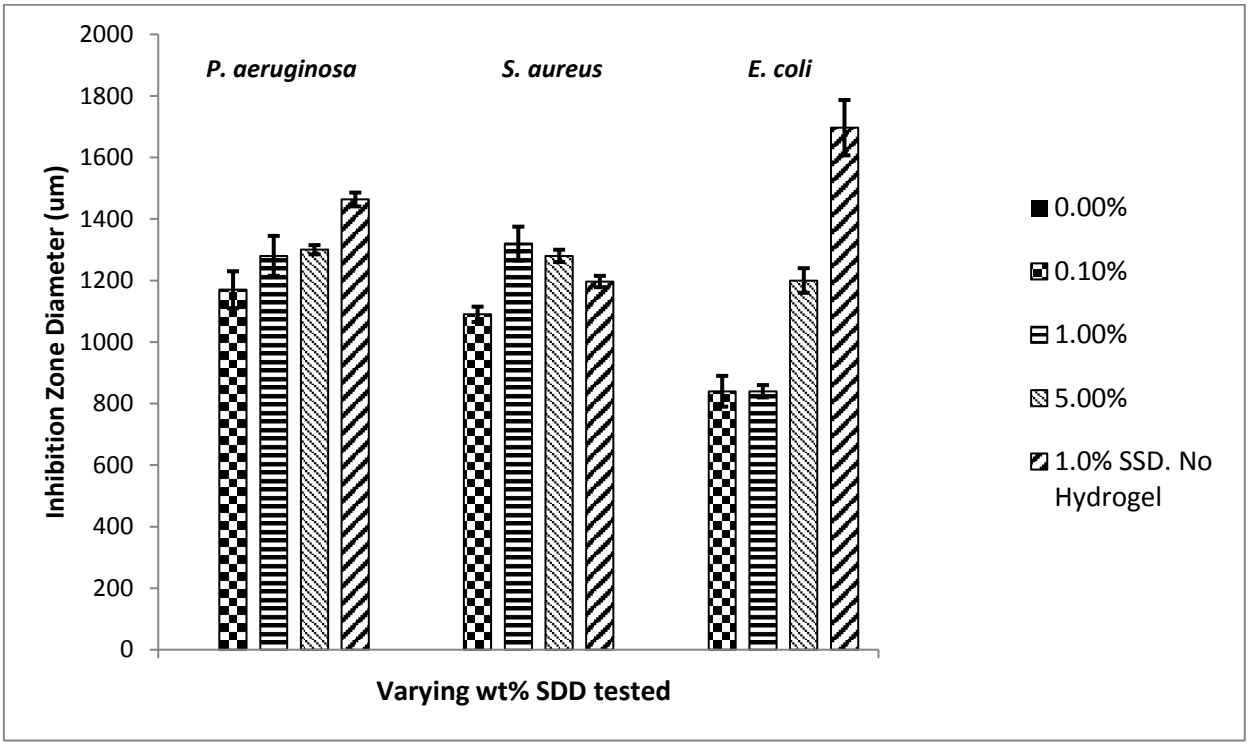

Figure 6. Disk Diffusion zones after 72 hours on plated samples. Zone diameters were taken using a Vernier Callipers. Legend on right refers to w/v% of SSD per hydrogel sample. Control hydrogels containing 0.0% SSD showed no zones of inhibition. Zones of inhibition for *E. coli* were smallest in diameter while SSD demonstrated inhibition in all cases where present. "1.0% SSD No hydrogel" represents SSD in aqueous solution plated on filter paper against bacterial samples without hydrogel carrier.

From the data presented in Figure 5, it is clear that the presence of silver sulfadiazine inhibited growth of *P. aeruginosa* in all hydrogel samples excluding control sample containing no SSD. Note, image appears as though only one sample present in 0.0% concentration; however, hydrogel samples containing no SSD were transparent and therefore not optically visible in these images due to transparency. From **Figure 5**, it

is clear that the presence of SSD in small amounts (ranging from 0.1% - 5.0% w/v) was sufficient in all studied cases to prevent bacterial growth. Bacterial strains: *P. aeruginosa, S. aureus* and *E. coli* were all studied and corresponding images presented in supporting information. Further studies will be undertaken to optimise the dosage with respect to inhibition of growth, SSD dispersion and cell viability.

From the data presented in **Figure 6**, we observe the numerical quantification of the zones of inhibition of hydrogels with loadings of 0.0, 0.1, 1.0, 5.0 % w/v SSD and a positive control of 1.0% SSD in aqueous solution (no hydrogel) plated on filter paper. It is clear that the presence of SSD is required to cause bacterial inhibition. The negative control for this study is the hydrogel material containing 0.0% SSD loading which presents no zone of inhibition. The positive control for this study is the 1.0% SSD plated on filter paper which presents clearly defined zones of inhibition. When we consider the hydrogel loaded with SSD, we observe zones of inhibition that are comparable with those of the positive control. In all hydrogel cases, loading of SSD in dosage of 0.1% w/v presents zones of inhibition that are smaller than those of 1.0 and 5.0% w/v loadings. There was no significant difference in the zones of inhibition for 1.0% and 5.0% w/v SSD loaded hydrogels when tested on bacteria *S. aureus* and *P. aeruginosa*. However, when hydrogels containing 1.0% and 5.0% SSD w/v loadings were tested on *E. coli*, the 5.0% w/v loading performed significantly better creating a larger zone of inhibition. It is noted that when this 5.0% w/v SSD loaded hydrogel was tested against *E. coli,* it presents a zone of inhibition that is no greater in size than the zones of inhibition for 1.0% and 5.0% w/v loadings when tested against *S. aureus* or *P. aeruginosa.* This data suggests that *E. coli* may be more resistant to SSD than *P. aeruginosa* and *S. aureus*, thus requiring a larger dosage.

3.3. Diffusion Studies

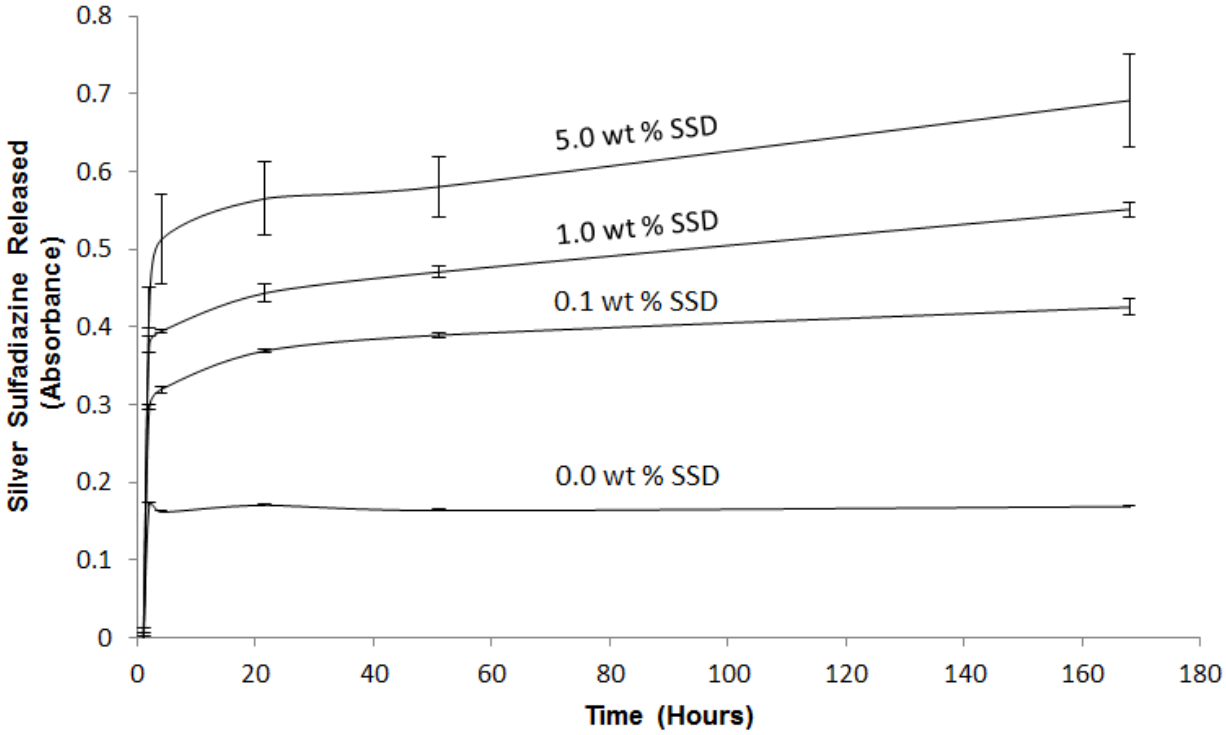

Figure 7. Plot of silver sulfadiazine (SSD) release (absorbance) measured by UV-vis spectroscopy against time (hours).

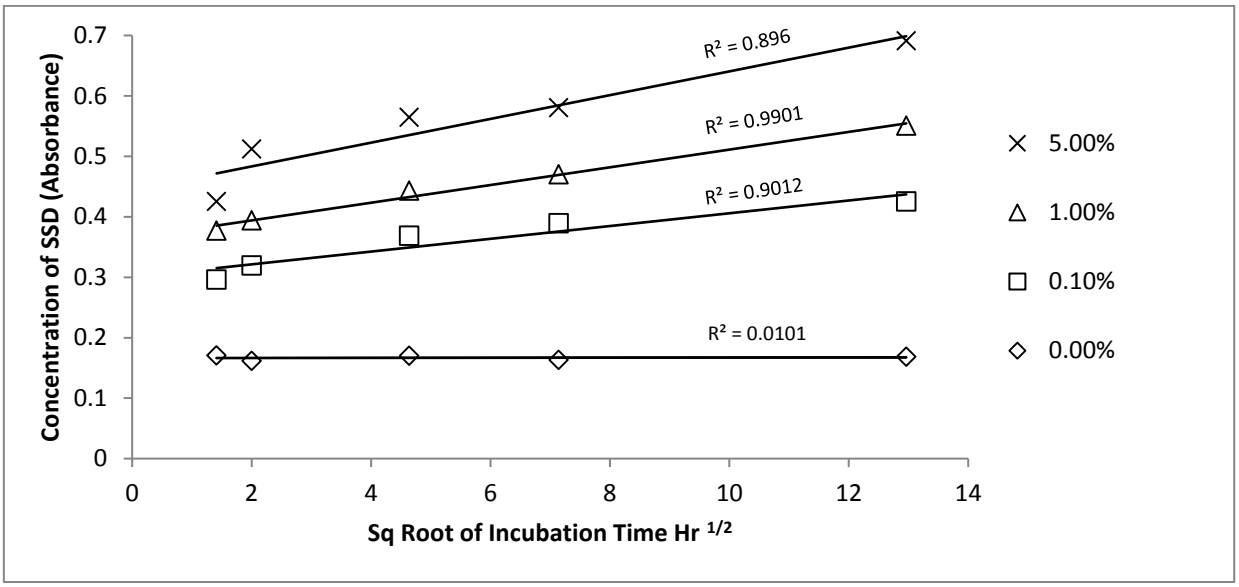

Figure 8: Plot of silver sulfadiazine (SSD) concentration in hydrogel sample against the square root of incubation time.

Diffusion studies were undertaken as a function of absorbance over time using spectroscopy. Prior to diffusion study we identified the optimum wavelength for observing the presence of SSD. This study identified 290nm as the most appropriate wavelength for SSD identification. From the above diffusion studies (**Figure 7**) it is clear that the SSD is released from these hydrogel samples in a controlled and predictable manner (increasing concentrations of SSD results in a faster and higher level of SSD release). This SSD release study demonstrates an initial burst release of sufficient quantities of SSD to create strong adjacent antibacterial effect (particularly beneficial in infected wound cases). The same step leads to a slow prolonged release thereafter which would allow for good surface compatibility after the initial hours. This initial SSD burst release from the hydrogel also helps to avoid possible risks or potential of resistance and tolerance of bacterial cells to Ag [41] [42], which might occur in a constantly low silver releasing environment.

Furthermore, increasing concentration of SSD within 0 – 5% w/v range does increase both the burst release rate and the amount of SSD release over a controlled period. This demonstrates that these

hydrogels could be tuned for controlled release of predictable drug levels. Furthermore this study shows that this hydrogel system steadily releases SSD beyond a time point of seven days. A recent study found that three commonly used silver based dressings (ACTICOAT™ Flex 3 (Smith & Nephew, Milan, Italy), Mepilex® Ag, Mölnlycke Health Care, Gallarate, Italy and ACTISORB® Silver 220 (Johnson & Johnson, Rome, Italy) guarantee sustained antimicrobial action for 3, 7 and 7 days respectively[43]. This demonstrates that our hydrogel system has potential to compete in this aspect with currently available silver based dressings.

When SSD absorption is plotted against the square root of incubation time (**Figure 8**), a linear relationship is obtained (except for the initial stages of soaking). Figure 9 shows that the samples containing SSD present the trend-line with a high goodness of fit R^2 values (0.896, 0.9901 and 0.9012). This is supported by literature[44] and indicates that the release of SSD is controlled by inter-diffusion of the particles within the hydrogel[45]. This inter-diffusion is an important mechanism to confirm as it demonstrates that the release profile is tuneable and predictable based on the concentration of SSD loaded initially and the structure of the hydrogels.

3.4. Cytotoxicity Investigation

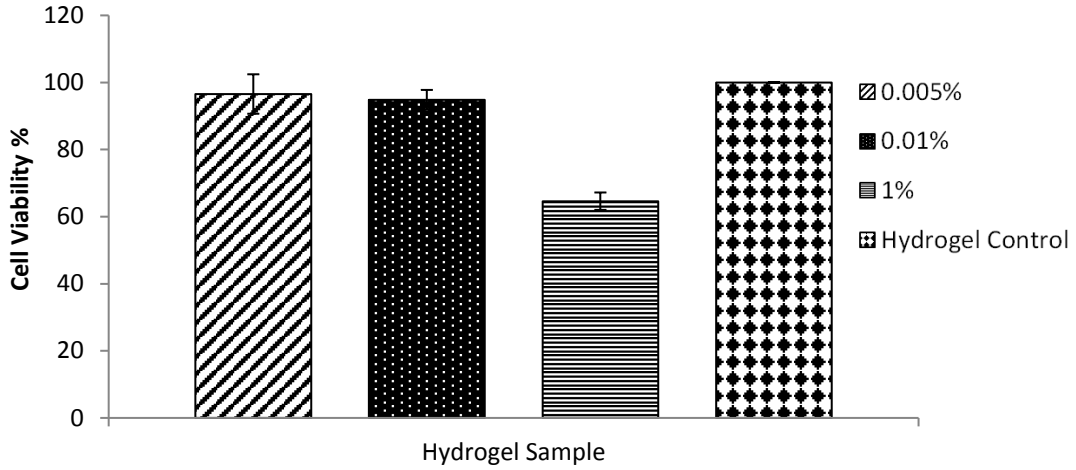

Figure 9. Cell Viability day 1. 0.005%, 0.01%, 1.0% silver sulfadiazine (SSD) hydrogels and 0.0% control hydrogel.

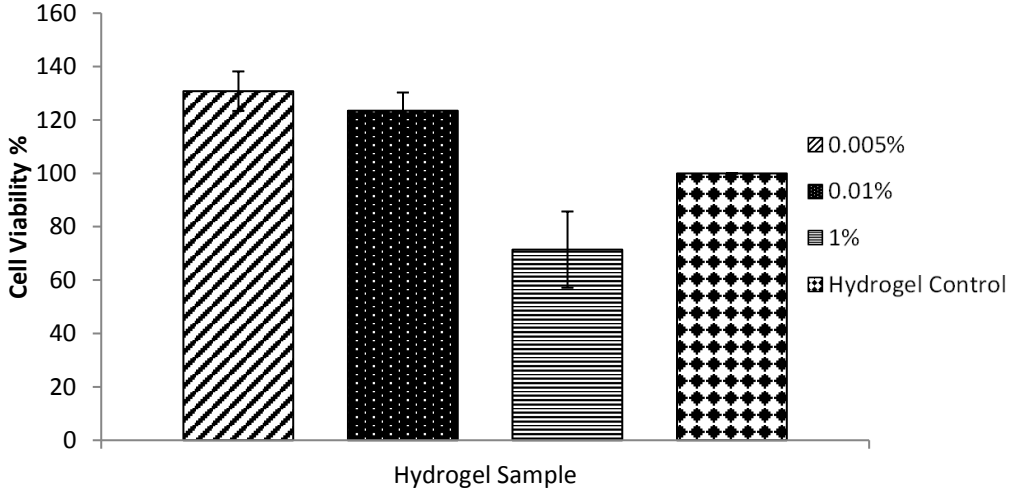

Figure 10. Cell Viability day 3. 0.005%, 0.01%, 1.0% silver sulfadiazine (SSD) hydrogels and 0.0% control hydrogel.

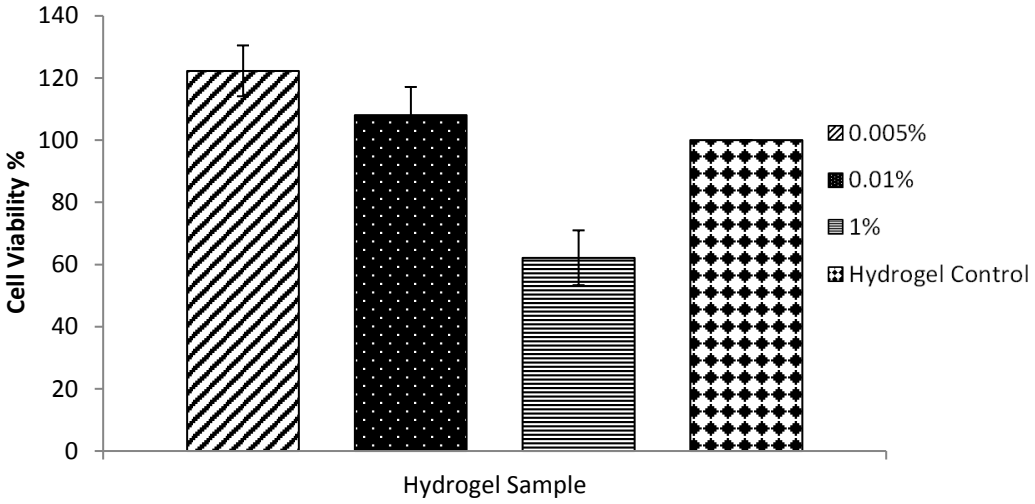

Figure 11. Cell Viability Day 7. 0.005%, 0.01%, 1.0% silver sulfadiazine (SSD) hydrogels and 0.0% control hydrogel.

In terms of the cell viability evaluation, hydrogel samples containing SSD of 0, 0.005, 0.01 and 1.0% w/v were tested using a standard Alamar blue assay on Adipose Derived Stem Cell's (ADSC's). The concentration of SSD was chosen at a lower level range for this study because it quickly became apparent that a higher concentration of SSD was toxic to these cells. Control hydrogel sample did not reduce the cell viability at day 1 (**Figure 9**), day 3 (**Figure 10**) and day 7 (**Figure 11**). Promisingly, the tests using SSD at 0.01 and 0.005% w/v dosage did not significantly reduce cell viability and cell proliferation either as observed by Day 7. However, the 1.0% w/v SSD loading resulted in a reduction in cell viability at Day 1. This can be explained by the toxicity caused by the initial burst release of SSD upon initial introduction of hydrogel samples to cells. At Day 1 cell viability is reduced to 64.61%. However, at Day 3 (Cell viability: 71.42%) and Day 7 (Cell Viability: 62.18%). Cell viability figures did not drop significantly over prolonged time, suggesting a high burst release being a contributory factor to early cell mortality. Interestingly, hydrogel samples containing low levels of SSD (0.01 & 0.005%) in figure 11 & 12 demonstrated increased cell proliferation at Day 3 and Day 7 with respect to the control hydrogel. This proliferation could be attributed to a friendlier hydrogel environment created at a low SSD concentration

whereby, an environment of sufficient antibacterial activity prevents bacterial growth without adversely impacting cellular activity thus providing a more ideal environment for cellular proliferation.

4. Discussion

One of the primary aims for the treatment of wounds is to prevent infection and then to promote proliferation of epithelial cells [46]. Therefore a wound dressing must find a balance between the antibacterial efficacy and cytotoxicity[47]. A moist environment is most suited for epithelialization and healing but this environment cannot become compromised with infection and therefore must actively inhibit bacterial growth. With this in mind, providing sufficient antibacterial efficacy to prevent bacterial growth but not to the extent that regenerative processes are hampered by aggressive treatments. New bioactive hydrogels such as cell laden hydrogels and gene delivery hydrogels require a good micro-environment that is free of infection to function effectively in the treatment of hard-to-heal chronic wounds. Therefore, antimicrobial hydrogel wound dressings should not simply aim to perform better than direct topical application of antimicrobial agent in terms of killing bacteria. It would be highly advantageous for the antimicrobial gel to contain low levels of antimicrobial agents (such as SSD) and release SSD in a controlled and sustained manner thus exhibiting low toxicity and minimized side effects. Moreover, antimicrobial hydrogels also have the advantage of being easily administered with a known dosage and forming gel in-situ. This antimicrobial gel can be used to deliver cells to the wound sites because of its cyto-compatibility.

A poly (ethylene glycol) PEG based copolymer was developed for the purpose of the hydrogel synthesis owing to its controllability, reproducibility and suitable chemical structure for hydrogel formation[48]. A PEGDA-PEGMEMA copolymer and subsequently 3-D hydrogel matrix were successfully created and optimised in combination with thiol modified Hyaluronic acid acting as the cross linking agent. The hyperbranched structure provides multiple acrylate functional groups which allow more effective crosslinking to form a network structure with a higher crosslinking density so that to tailor the release profile of the bioactive molecules encapsulated [34]. We applied this hydrogel as a drug delivery system to

inhibit the growth of the bacteria: *S. aureus*, *P. aeruginosa* and *E. coli*. After consideration of a number of antibacterial agents, silver sulfadiazine (SSD) was selected. SSD was loaded into these hydrogel samples successfully at varying concentration.

A standard Kirby-bauer disk diffusion test[49] was adapted and used to evaluate SSD encapsulated hydrogels on their bacterial inhibiting capability. Results were encouraging and showed that the growth of tested bacterial strains was thoroughly supressed when low concentrations of SSD were used. Upon analysis of these results and comparison of the zones of inhibition achieved from antibacterial agent concentrations of 0.0, 0.1, 1.0, 5.0% we noted that diffusion of SSD was a key factor in the zone of inhibition and that the direct contact and immediate areas surrounding it effectively inhibited growth of all three bacterial strains. Additionally, in an attempt to model and assess the release profile of this hydrogel system, release assays were performed in a PBS buffer over extended periods of time using SSD in the previously studied 0.0, 0.1, 1.0 and 5.0% w/v concentrations. This allowed for a detailed representation of the diffusion profile that can be expected from these hydrogels. The study demonstrated a burst release effect initially of SSD owing to the presence of particles on the hydrogel surface. The study also revealed that increased concentrations of SSD resulted in a faster rate of diffusion with higher SSD released over a more prolonged timeline.

Finally a simple biological assay in the form of Alamar blue cell viability assay was performed to evaluate the effect of SSD loaded hydrogels on the survival and proliferation of ADSC's. The test was performed at lowered concentrations as it became apparent that high (> 1.0% w/v) SSD concentration was toxic to cell viability. This test confirms that levels of SSD concentration above 1.0% w/v SSD in these hydrogels can be toxic to cells. This can in part be related to the initial burst release of SSD being overwhelming as the cell viability decreases initially (Day 1) for 1.0% w/v SSD and remains relatively stable through Day 3 and Day 7. The antibacterial assessment by means of a Disk Diffusion study of this hydrogel has confirmed that this low concentration (0.1 & 1.0% w/v) can have significant antimicrobial potency and can protect a wound from pathogenic development for extended time periods. This study

also suggests that at lowered concentrations (0.1% & 1% w/v), these hydrogels can be used to inhibit bacteria without un-acceptable cell viability; however, burst release should be kept mindful.

5. Conclusions

In conclusion, a new dressing in the form of a composite hydrogel was successfully synthesised from the copolymer PEGDA-PEGMEMA and thiol modified hyaluronic acid as a cross linker, while silver based antimicrobial agent SSD was encapsulated. This new dressing demonstrates powerful antibacterial efficacy against three strains of the prime concern (*S. aureus*, *P. aeruginosa* and *E. coli*) in the fight against resistant bacteria.

Early cytotoxicity assessment of the dressing has revealed that increased concentrations of silver sulfadiazine adversely effects ADSC proliferation but that low concentrations can be used effectively without causing unacceptable cell mortality. However; burst release of SSD in contact with cells is an area for concern to keep mindful. Of these concentrations we determined that 1.0% w/v SSD concentration was most suitable for this application as it effectively inhibited bacterial growth of tested pathogens *S. aureus, P. aeruginosa* and *E. coli* while presenting an acceptable mortality to ADSC's. Cell viability did highlight the concerns around the burst release effect and warrants this as an area of concern for future studies. Furthermore, this 1% w/v formulation demonstrated a release profile with high burst rate in the beginning but a controlled slow release rate up as far as a 7 days tested period. We think that this release profile should be predictable and controllable given that release is governed by inter-diffusion of particles after initial burst release of surface SSD. This burst release aspect presents a design challenge for this hydrogel loading that we will seek overcome in future studies. Further confirmation of the drug loading limitations will be optimised in a subsequent study.

This research presents relevant information indicating that our new hydrogel system can be readily combined with current drugs/therapies (SSD) to prevent growth of bacteria. This partnership works well and our study into the systems release profile suggests a predictable, controllable release pattern. This

early study will be continued into further detailed optimisation and investigation into long term response/release and performance against infectious pathogens as well as detailed cell proliferation studies.

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Supporting Information description:

(Co-Polymer Synthesis andCharacterisation): Detailed information is provided on polymer synthesis by RAFT polymerisation, characterisation of polymers by NMR, GPC for quantification of polymer branching degree,vinyl groups and molecular weight. (**Bacterial studies):**Bacterial strain growth curve quantification, supporting images for inhibition zones of the hydrogels containing SSD tested on plates of *S. aureus*, *P. aeruginosa*, *E. coli. (***SSD Characterisation):** Zeta particle size analysis, Zeta potential analysis. (**Hydrogel data):** Supplementary information on the method for the preparation of hydrogel samples including reactants. (**Release study):** Wavelength study for identification of SSD using absorbance,

Contents

7. Notes and references

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/c000000x

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