Journal of Materials Chemistry B

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/materialsB

Multi-modal delivery of therapeutics using biomaterial scaffolds

S. Browne[A] and A. Pandit[A]*

Functionalisation of biomaterials with therapeutic moieties (proteins, drugs, genes) is a pre-requisite to tissue regeneration and restoration of function following injury or disease. However, up until now, single-factor delivery has not proven to be clinically efficacious, most likely due to the complex nature of pathological states. In this regard, strategies that respect the complex nature of disease can prove successful, paving the way for the delivery of several factors to modulate several stages of the pathology over time. Biomaterials offer opportunities to deliver multiple therapeutics in a temporal manner (multi-modal release) using a number of strategies. The importance of these strategies will be described, as well as the methodologies used to achieve multi-modal release. Furthermore, strategies to engineer more programmed and responsive biomaterials as multi-modal delivery systems will be explored.

1 Introduction

The use of biomaterials in the fields of drug delivery and regenerative medicine is now well established.[1–7] The initial use of biomaterials as rigid implants to mechanically stabilize damaged tissue or organs has now evolved to materials capable of restoring function following injury, promoting regeneration and integration in the body. This therapeutic role is often aided by engineering specific functional entities into the biomaterial. The range of functional entities includes proteins, genes, antibodies, cells, or combinations thereof. Biomaterials can be fabricated depending on the application, in the form of a sponge,[8–11] hydrogel,[12–16] fibre[17,18] or microparticles,[19–25] can act as a reservoir of the therapeutic, maintaining its local concentration, protecting it from degradation and prolonging release of the therapeutic.

Typical therapeutics utilized to improve the integration of biomaterials into the body are either anti-inflammatory or pro-angiogenic. Anti-inflammatory therapies aim to minimize the foreign body response to biomaterials,[26] which can result in rejection and fibrous capsule formation[27–30], while pro-angiogenic therapies aim to increase vascularization of the scaffold and thus integration into the body, In addition, survival of any implanted cells can be enhanced by this strategy.[31] More recently, biomaterials have been used to deliver anti-inflammatory and pro-angiogenic therapies to pathologically inflamed and ischemic tissues, respectively, rather than for modulation of a prolonged foreign body response. In addition, various therapeutics (anti-apoptotic, osteogenic, chondrogenic, neurotrophic etc.) specific to the tissue of interest and the specific disease state being treated, have been delivered via biomaterials. In each case, biomaterials tend to increase the effectiveness of therapy over bolus injection by extending the time of release and efficacy of the therapeutic. However, it is worth considering that despite the increased efficacy of a therapeutic when combined with a biomaterial delivery system, the therapy is dependent on the activity of the therapeutic itself and its intervention in the disease pathway to promote recovery. That is: will delivery of that single therapeutic result in complete resolution of the pathology? This is a biological rather than a materials question, and requires a thorough understanding of the pathology associated with the disease and tissue being targeted. In most cases, it is quite rare that a single intervention will significantly alter and resolve a complex disease state. Such cases do exist, specifically in genetic diseases such as recessive dystrophic epidermis bullosa (RDEB), a blistering skin condition in which patients are incapable of producing the protein collagen type VII.[32,33] In this case, correction of this gene should in theory overcome the disease state. However, treatment in the majority of diseases a more holistic approach is necessary and this requires an understanding of the overall pathology.

This review outlines the necessity for multi-modal delivery strategies to target specific pathways using biomaterials. In addition, mechanisms to increase the responsiveness of biomaterial delivery systems to react to injury and stimuli in a temporal manner will be elucidated.

2 Multi-modal delivery from biomaterials

Wound healing is driven by various factors that are present at distinct time points in the regeneration process.[34–36] Following tissue injury and after the formation of a clot, there is an initial inflammatory phase, characterized by the presence of cells such as pro-inflammatory macrophages and neutrophils. Subsequently, there is an increased production of radical oxygen species (ROS) and matrix metalloproteinases (MMPs).[37] Gradually, this inflammatory response subsides and is replaced by a proliferative phase during which increased angiogenesis and extracellular matrix (ECM) deposition occurs, and there is a shift in macrophage phenotype from a pro-inflammatory to a more regulatory phenotype. There is also a reduction in capillary formation as the wound enters the remodeling phase, with constant re-organization of the matrix through the activity of fibroblasts, MMPs and tissue inhibitors of matrix metalloproteinases (TIMPs). It is apparent that biological phenomena such as angiogenesis, inflammation and tissue remodeling are complex and interlinked processes that are controlled in a spatiotemporal manner. That is, the appearance of various factors in the local microenvironment is carefully orchestrated and follows a defined path. Thus, to assume that these processes can be manipulated and re-capitulated by delivery of a single factor is to oversimplify complex biology. In fact, this phenomenon explains the failure of single factor delivery systems in the clinic. For instance, delivery of vascular endothelial growth factor (VEGF) alone often leads to the formation of immature leaky blood vessels characteristic of tumours.[38] In addition, these vessels are often prone to regression over time, compromising the perfusion of the tissue. In comparison, systems that deliver multiple growth factors have led to increased maturation of newly formed vessels. [39] It is evident, therefore, that there is a clear need to engineer biomaterials that can control the release of factors over time, in an attempt to control processes such as these. Materials capable of controlling the release of multiple factors such that there is a differential release of specific factors over time can mimic the spatiotemporal nature of biological processes.

2.1 The need for multi-modal delivery

The use of single factor delivery to treat various pathological and disease states thus far has proven insufficient from a clinical standpoint. This is hardly surprising given the complexity of many disease states. For instance, if one examines the normal wound healing response, there is an initial inflammatory phase, followed by a proliferative phase and finally the remodeling phase. During compromised wound healing, such as with diabetic wounds, there is dysregulation in all of the phases.[40] In addition, with the reduced endothelial nitric oxide synthase (eNOS) activity and nitric oxide (NO) availability due to the increased production of ROS, there is diminished angiogenesis, and issues with inflammation as well as changes in ECM composition.[41,42] When designing a therapy, one has to carefully consider the target pathological stage, and determine the stage of the primary cause of impaired wound healing. In the case of diabetes, this would mean deciding whether to target the chronic inflammatory state, attempt to reduce ROS expression, try to overcome the lack of sufficient angiogenesis or

attempt to modulate the changes in ECM composition. However, it is apparent that in order to deal adequately with the pathological state, a multi-faceted approach is necessary. This is true of not only diabetes but also of a number of other conditions such as critical limb ischemia (CLI) and myocardial infarction (MI). But the delivery of multiple factors with antagonistic functions at one time can complicate the process, given that processes such as inflammation and angiogenesis are so intricately and closely linked that they follow a very distinct time-course. Thus not only is the delivery of multiple factors necessary but it must be controlled in a temporal manner, allowing for changes from one phase of the pathological condition to the next. For this reason, biomaterials that can control the timing of delivery of multiple factors relative to each other have therapeutic value.

In addition to their use as therapeutic delivery systems, biomaterials that can modulate the temporal release of multiple factors are of biological significance. Using biomaterials, which can impart control over the temporal release of factors, are useful tools that permit an investigation of cross-talk between multiple factors. For example, scaffolds with these multi-modal release properties can be used to study interactions between various angiogenic factors to determine how mature and stable blood vessels are formed in all four dimensions. By utilizing these materials in a systematic way, the mechanisms behind complex processes may be elucidated, and more relevant and efficacious therapies developed.

2.2 Biomaterial strategies to achieve multi-modal delivery

The current paradigm in tissue engineering is the use of biomaterials as reservoirs to extend the release of therapeutics. These strategies involve the use of protein therapeutics, nucleic acids or drugs. However, from a biomaterials standpoint the parameters of significance are the physico-chemical properties of the therapeutic, and its optimal combination with a biomaterial. Typically, therapeutics may simply be loaded and physically entrapped within scaffolds. The therapeutic is subsequently released over time through a combination of diffusion and degradation of the scaffold. This has proven moderately successful with both natural and synthetically-derived scaffolds. However, especially for synthetic scaffolds, there is no significant control over the release of the therapeutic given that the degradation is not enzymatically driven. The only control is that of the concentration and physical form of the material. With natural materials, enzymatic degradation will trigger release of loaded therapeutics. However, this property can be engineered into synthetic scaffolds.[43,44] In addition to enzyme-mediated degradation, binding sites for therapeutics may be engineered into material systems.[45,46] Another possibility is the modification of the therapeutic or the use of a variant such that it has a sequence that will bind to the natural scaffold. This has been shown with VEGF and fibrin matrices previously.[47] While these strategies offer control over release of a single-loaded therapeutic, alternative strategies are necessary to control the release of multiple factors in a temporal and controlled manner in an attempt to re-capitulate natural developmental and regenerative processes.

2.2.1 Scaffolds and hydrogels

Biomaterials in the form of porous scaffolds and hydrogels are the most common scaffolds used in tissue engineering. Typically therapeutics are physically incorporated into the biomaterial either during or after the fabrication process. Delivery of VEGF and FGF-2 from a PLGA scaffold (or bridge) has been reported to promote angiogenesis in a rat spinal cord hemi-section model to treat the ischemia associated with the injury.[48] Dual delivery of the growth factors was observed to increase endothelial cell infiltration and blood vessel formation. This resulted in a trend towards increased neurite ingrowth into the implanted bridge. Thus, delivery of factors to promote angiogenesis may help to overcome the ischemic environment that negatively affects regeneration. However, in this study the use of single factor delivery was not used as a control, and thus it is impossible to say definitively that multiple factor delivery is more powerful than either single factor delivery, or to interpret the interaction between the factors as a function of release from the biomaterial.

Layered PLG scaffolds containing distinct regions of VEGF and anti-VEGF were constructed to create spatially restricted angiogenic regions.[49] It was observed that increased angiogenesis occurred in the VEGF layers while minimum angiogenesis was observed in the anti-VEGF layers, as the anti-VEGF blocked the activity of the VEGF as it diffused away from the VEGF layer. The layered scaffold was assessed in an ischemic limb model and it was observed that scaffolds containing both the VEGF and anti-VEGF layers produced greater perfusion of the limb than the blank scaffold. The importance of this study is that it spatially restricts the activity of a factor by delivering an antagonist to restrict its zone of activity. The region of action can be increased or decreased by altering the doses of VEGF and anti-VEGF, respectively.

An alginate affinity-based system was utilized to deliver insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF) in a sequential manner to the infarcted myocardium.[50] This was found to increase formation of blood vessels and reduce apoptosis in the myocardium, with reduced fibrosis also being apparent. While this study illustrated the potential advantages of a delivery system compared with a saline injection, it is difficult to decipher whether either growth factor delivered via a biomaterial will be sufficient as these groups were not included in the study. Similarly, an alginate system was used to enhance the regeneration of ischemic muscle[51] with VEGF and IGF delivered along with myoblasts. It was observed that there was a reduction in the defect area at six weeks. Increased angiogenesis and perfusion were seen, while there was an improvement in the tetanic force generated in the anterior tibialis muscle when both factors were delivered compared with either factor alone or bolus delivery of the factors. The use of a biomaterial system thus ensured a more extended presence of the delivered factors in the local environment.

Alginate-sulfate has been used to sequentially deliver three factors to induce neovascularization.[52] The use of alginate-sulfate allows for the loading of heparin-binding factors, or VEGF, PDGF-BB and transforming growth

factor-β (TGF-β) in this case. An initial burst release of VEGF is followed by a more prolonged and delayed release of PDGF-BB and TGF-β, similar to that which occurs in normal blood vessel formation. When using only alginate, no difference was observed in the release profile of these factors, with release also occurring much quicker for each of the factors. *In vivo*, this differential release profile proved capable of increasing blood vessel density after one and three months. The timing of the release of multiple factors, and subtle changes in material characteristics can thus have an effect on biological processes.

A poly (ethylene glycol) (PEG)-maleimide gel was formed with RGD sequences and growth factor binding domains, with the ability to control the growth factor release via protease-cleavable linkers.[53] Dual delivery of HGF and VEGF was found to improve function in the rat myocardium following ischemia/reperfusion. However, there was no difference in the release profile of the growth factors as they were released simultaneously. Time-delayed release of one of the factors relative to the other, or incorporation of a separate factor to be differentially released can further improve this system. A brushite-chitosan system was used to control the release of angiogenic factors. VEGF and PDGF were delivered in a bone defect model.[54] The authors compared the *in vitro* and *in vivo* release profiles using radiolabelled growth factors. The same patterns were observed with minimal differences between *in vitro* and *in vivo*. It was found that the combination of growth factors greatly increased the formation of new bone than occurs with either growth factor alone, due to increased blood vessel formation and maturation.

Single-walled carbon nanotubes have been developed for use as biomedical sensors. However, inflammation results from implantation and compromises the function of the sensor. Thus, carbon nanotube sensors have been coated with a PEG hydrogel loaded with dexamethasone (DX) and VEGF. In a chick embryo chorioallantoic membrane (CAM)[55] the release profile was assessed and revealed that the DX release was quicker than the VEGF. Using both DX and VEGF, the therapeutic index, defined as the ratio of vasculature density to inflammatory cell density, was higher than that of the control. However, there was little difference between this group and the VEGF alone group, while the group delivering DX in bolus form followed by sustained release of VEGF had an improved therapeutic index. This may be due to an interaction between the functions of DX and VEGF. Thus, it may be that a more precise control over the release profiles, such that there is a greater difference between DX and VEGF release, would result in an improved outcome. Similarly, a hydrogel formed from 2-hydroxy-ethyl methacrylate, N-vinyl pyrrolidinone, and PEG was loaded with DX and VEGF and used as a coating to reduce the foreign body response to implanted glucose sensors.[56] The VEGF increased angiogenesis and inflammation, while the DX had the opposite effect. Thus a system that can tune the release of the two therapeutic components of the system is useful in a scenario in which both inflammation and angiogenesis must be controlled.

2.2.2 Micro- and nanoparticles

Wang et al developed an anti-cancer strategy using a nanocapsule delivery system. To treat a subcutaneous tumor model in mice, nanocapsules were used to sequentially deliver an agent to disrupt the vasculature, combretstatin A4 (CA4), and an anti-cancer agent, paclitaxel (PTX).[57] Sequential release was achieved by the slow hydrolysis of the ester linkage between the PTX and the poly (lactic acid) (PLA) polymer, which delayed its release more than that of the CA4. It was found that sequential delivery of these factors using the nanocapsule system resulted in reduction in tumor volume and 100% survival of the treated mice. A similar anti-cancer strategy was developed using liposomes decorated with low-density lipoprotein receptor-related protein receptor (Angiopep-2) and neuropilin-1 receptor (tLyP-1). Yang et al. delivered VEGF siRNA and PTX to subcutaneous xenograft tumor models in mice. It was found that dual delivery resulted in reduced levels of VEGF, increased apoptosis and a reduction in tumor volume. This was observed both when the liposomes were delivered directly to the tumor and when delivered intravenously.[58]

Dual delivery of siRNA targeting IL-10 along with a pDNA vaccine encoding for hepatitis-B surface antigen (gWizHBsAg) using poly (ethylenimine) (PEI)-PLGA microparticles was observed to tune the immune response in a mouse *in vivo* model.[59] This system however was designed to co-deliver the nucleic acids to the same cells, and thus there was no sequential release. This system can be modified to alter the release profile, allowing for sequential delivery of nucleic acids.

Cittadini et al. used gelatin microspheres to produce an initial burst release of IGF-1 followed by a sustained release of VEGF.[60] It is not clear how this release profile is achieved, although it appears simply due to an increased natural affinity between the gelatin and the VEGF over the IGF-1. Dual-delivery of both IGF-1 and VEGF together had complimentary effects: reducing inflammation, increasing angiogenesis, and reducing the infarct size. Alginate-albumin particles have also been used in a similar way to deliver angiogenic factors to the myocardium in a chronic heart failure model.[61] The interaction between FGF-2 and HGF when they were delivered simultaneously was examined, and its ability to promote angiogenesis. It was revealed that delivery of both factors increased not only the number of blood vessels but also the number of mature vessels. This resulted in an improved functional performance of the heart. A system that modulates the release of one factor relative to another could be used to study the spatiotemporal relationship between the factors, which in turn could lead to improved efficacy.

PLGA microparticles were used to deliver combinations of angiogenic factors to induce angiogenesis in a CLI model.[62] Endothelial progenitors were also delivered to aid in the angiogenic process. PLGA particles formed using a double-emulsion process were loaded with VEGF, HGF or Ang-1, and used in various combinations in an *in vivo* matrigel plug assay. It was observed that delivery of all three factors was superior in terms of blood vessel

formation to dual delivery of VEGF and HGF, which itself was superior to delivery of VEGF alone. In the ischemic limb model, delivery of VEGF, HGF and Ang-1 along with endothelial progenitors increased perfusion of the ischemic limb when injected into the muscle. The PLGA particles were designed to release the growth factors over a period of up to two weeks, with any difference in release likely to be minimal. However, the fact that microparticles were used allows for the possibility of their incorporation into a scaffold or hydrogel to further tailor the release, or to modulate the release of factors relative to one another and thus increase the degree of control over their interaction. Collagen microspheres were loaded with bFGF and HGF and used to induce angiogenesis in a CLI model.[63] *In vivo* release studies showed that when bFGF and HGF were delivered simultaneously to the limb increased perfusion of the limb resulted than that of either factor alone at a higher dose. Thus the synergism of the two factors results in a greater effect than with either factor alone.

2.2.3 Fibrous scaffolds

A cellulose acetate hollow fibre system was used to prolong the release of angiogenic factors and was tested *in vivo* using a matrigel™ plug assay.[64] It was found that delivery of VEGF followed by delivery of sphingosine 1-phosphate (S1P) resulted in the formation of a mature vasculature compared with either factor alone or both factors simultaneously. While this does prove the necessity for sequential delivery, the delivery system was not ideal. The hollow fibres were implanted and then injected with the angiogenic factor every 24 hours, with VEGF injected for the first three days and S1P subsequently injected for three days. Thus, while the system is not suitable as a therapeutic delivery system, it has shown its usefulness as a tool for studying the interaction between various factors. This was also observed when the same system was used to study the interaction between bFGF and PDGF in a subcutaneous matrigel™ plug assay.[65] In this case, the switch from bFGF to PDGF was on the third day. It was observed that delivery of bFGF followed by PDGF resulted in greater blood vessel formation and maturation compared with either factor alone, both factors delivered simultaneously or PDGF followed by bFGF.

Electrospun fibres may also be loaded with different factors during the fabrication process, negating the need for continual application of therapeutics following implantation as in previously described studies. This has been shown by Man et al., who fabricated electrospun fibres with poly (vinyl pyrrolidone)/bovine serum albumin as the core fluid and poly (ε-caprolactone) (PCL) solution as the sheath fluid. TGF-β1 was loaded within the core fluid and a bone marrow-derived stem cell (BMSC) affinity peptide (E7) was attached to the PCL shell. Release studies revealed that the TGF-β1 was released gradually over 21 days after an initial burst over the first five days. *In vitro* studies show that the presence of the E7 peptide increased the attachment and proliferation of the BMSCs, while the gradual release of TGF-β1 promoted cartilage differentiation. Thus, this fibre-based scaffold shows potential to promote the attachment of stem cells and direct their differentiation, depending on the cytokine loaded within the scaffold.[66] Electrospinning can also be used to form differentially loaded membranes. Electrospun membranes were fabricated with VEGF and PDGF loaded in the inner and outer layers, respectively.[67] When implanted *in*

vivo, it was observed that the group delivering VEGF and PDGF allowed endothelial cells to attach to the lumen and smooth muscle cells to form a layer on the outside.

Fibrous scaffolds composed of poly (DL-lactide)–poly (ethylene glycol) (PELA) were loaded with either *p*VEGF polyplexes, basic fibroblast growth factor (bFGF) polyplexes or both, and implanted subcutaneously to observe the angiogenic effect.[68] Polyplexes were formed using the commercially available transfecting agent PEI. It was seen that delivery of both *p*VEGF and *p*bFGF together increased the blood vessel density at two and four weeks. However, there appeared to be very little difference in the release profiles, with any differences attributed to polyplex size and charge. This shows that release of two complimentary factors is superior to that of one factor; nevertheless the timings of release of each needs optimization.

2.2.4 Spheres-in-scaffold/hydrogel composite

The use of biomaterials to deliver multiple therapeutics has been investigated over a number of years.[69] The use of composite scaffolds with microspheres contained within a bulk scaffold (solid or hydrogel) has proven to be a very popular method to achieve differential release of factors. Biomaterial systems that are able to mediate the release of VEGF and platelet-derived growth factor (PDGF) so that VEGF is released much quicker than the PDGF have been designed. This was achieved by mixing lyophilized VEGF with PDGF within microspheres, which were then incorporated into the same poly (lactide-co-glycolide) (PLG) scaffold. It was observed that co-delivery of these factors in a temporal manner using a polymeric system resulted in the increased formation of mature and larger blood vessels. This utilization of a biomaterial to mimic the native angiogenic process shows the importance of the temporal delivery of factors, demonstrated by the fact that delivery of single factors with a biomaterial or both factors via bolus injection resulted in a lesser angiogenic response. To further emphasize the crucial temporal control over factors, a similar study utilised a bi-layered scaffold which had VEGF alone in one spatial domain and VEGF/PDGF delivered sequentially in an adjacent region.[70] Following implantation in the ischemic hind limbs of mice, it was observed that in the VEGF alone treated side, small immature blood vessels were formed, but in the region treated with a temporal combination of VEGF and PDGF, there were fewer blood vessels but these vessels were larger and mature, at both two and six weeks. An alginate gel system has been used in the myocardium to deliver these same two factors, VEGF and PDGF, in a sequential manner.[71] Increased vessel density was observed, along with an improvement in myocardial function. The differential release of the two factors, however, was not a result of physical entrapment within microspheres as before, but rather a result of differential affinity between the different growth factors and the alginate hydrogel. This was not as a result of modification of the material but rather an intrinsic property of the alginate and growth factors. These studies illustrate the ability to utilise the properties of materials for multiple, sequential release. Material properties may also be engineered into substrates and growth factors for single factor release. By engineering increased affinity for a specific factor for which

delayed release is appropriate, and non-modification of a factor which is required for an earlier/quicker release, multi-modal systems can also be constructed.

The delivery of more than two factors is also possible through biomaterial scaffolds. Using a PLG scaffold (microspheres and scaffold composite), the temporal presentation of multiple angiogenic factors was investigated. It was found that co-delivery of VEGF and angeopoeitin-2 (ANG-2) followed by PDGF and angeopoeitin-1 (ANG-1) using this system induced the formation of a more stable vasculature characterized by increased alpha-smooth muscle actin (α-SMA) positive vessels.[39] This emphasizes a number of important factors, the first of which is the significance of multiple factor release in biological processes. The second is the critical need of temporal presentation, as in this case the pro-angiogenic factors VEGF and ANG-2 are released first followed by the release of the pro-maturation factors PDGF and ANG-1. This ensures the formation of a stable vasculature, which does not occur with delivery of just VEGF and ANG-2.

To induce angiogenesis in the infarcted myocardium, an N-isopropylacrylamide (NIPAAm)-based thermally responsive hydrogel with poly (lactic-co-glycolic acid) (PLGA) microspheres were used to deliver basic fibroblast growth factor (bFGF) and IGF-1.[72] However, no added benefit was observed when these growth factors were added to the biomaterial compared with the biomaterial alone as the cytokines lose bioactivity over time in this system.

The PLGA particles previously described[59] were combined with a dextran hydrogel to create a sequential delivery system. Macrophage inflammatory protein-3α (MIP-3α) was loaded into the dextran hydrogel to be released faster than the PLGA particles and attract dendritic cells. The release of PLGA particles with IL-10 siRNA and a pDNA antigen was enabled as the dextran hydrogel degraded, enhancing the immune response by shifting the T cell response to a Th2 type response.[73] *In vitro* studies confirmed the ability of the system to attract dendritic cells and reduce the IL-10 expression illustrating the potential efficacy of the system to deliver chemokines and nucleic acids in a single system. The system can be altered to allow for the delivery of alternative combinations of chemokines and genes, enabling use of the system in a range of disease states and pathologies.

A synthetic thermoresponsive hydrogel system was developed by Nelson et al. based on NIPAAm. Through the incorporation of a protein-reactive methacryloxy N-hydroxysuccinimide (MANHS) group, increased protein loading and retention was achieved. By addition of a hydrophilic acrylic acid group, degradation of the hydrogel, and hence protein release, can be increased.[74] A further layer of functionality and control was achieved by adding protein-loaded PLGA microspheres to the gel system. As expected, release of protein from the gel occurred before release from PLGA spheres. This system allows precise control over the timing of release through the incorporation of protein reactive groups as well as cleavable sites. However, no biological functionality of a released protein was observed, with bovine serum albumin (BSA) used as a model protein. A similar sphere-in-gel

system using chitosan gel and gelatin microspheres was characterized *in vitro* and used to increase the osteoblastic differentiation of W-20-17 mouse bone marrow cells.[75] This composite chitosan/gelatin microspheres system used to sequentially deliver bone morphogenic protein-2 (BMP-2) and IGF-1 showed an increase in alkaline phosphatase (ALP) activity at five and seven days.

A composite poly (propylene fumarate) (PPF) scaffold with gelatin microparticles was used to deliver VEGF and BMP-2 in a critical-sized bone defect in a rat.[76] Dual delivery improved the bone formation at four weeks, but this effect disappeared at 12 weeks as the group treated with the combination of VEGF and BMP-2 was similar to the group treated with BMP-2 alone. A more controlled release strategy to optimize the interaction between the angiogenic VEGF and the bone-forming BMP-2 in a temporal manner may yield increased bone formation over BMP-2 alone. In a follow-on study to assess the effect of dose on bone formation, no difference was seen in bone formation following VEGF delivery at 12 weeks.[77] There was no obvious benefit of the delivery of VEGF and BMP-2 over the delivery of BMP-2 alone at 12 weeks. However, the authors speculate that further optimization of loading dose, growth factor ratio and release kinetics can result in improved bone formation. In contrast, another study, using VEGF and BMP-2 in combination, observed an increase in bone formation following dual delivery using PLGA microspheres in a PPF scaffold, surrounded by a gelatin hydrogel.[78] This system was implanted both subcutaneously (ectopic model) and in a critical sized defect model. However, this difference may also be due to the different time point used (eight weeks).

A collagen/fibronectin hydrogel with alginate microparticles embedded within it was used to increase the survival and therapeutic potential of transplanted endothelial cells.[79] VEGF and monocyte chemoattractant protein-1 (MCP-1) were delivered in a sequential manner and resulted in an increased number of blood vessels when compared with delivery of either factor alone. In addition, there was no increased inflammatory response detected at two weeks.

Wang et al. utilized a double-sphere-in-gel system to control the release of two factors, epidermal growth factor (EGF) followed by erythropoietin (EPO), in an attempt to stimulate endogenous stem/progenitor cells in a stroke model.[80] EGF was modified with a 5kDa PEG and encapsulated within PLGA particles while the EPO was encapsulated within bi-phasic particles consisting of PLGA coated with poly (sebacic acid) (PSA). Both of these types of particles were encapsulated in a hyaluronan methylcellulose hydrogel. The purpose of the hydrogel was two-fold: to retain the particles and growth factors in the local area; and to attenuate inflammation, an intrinsic property of high molecular weight hyaluronic acid.[81,82] It was observed that delivery of EGF and EPO through the composite delivery system improved recovery compared with that of vehicle alone or growth factor delivery via a pump, although the lack of delivery of EGF or EPO alone makes it difficult to determine the importance of dual delivery or the interplay between the two factors. The importance of relevant controls in these studies cannot be

underestimated as the effects of each individual component in the system need to be determined, along with the interaction between the factors.

The use of a PLGA sphere/alginate gel system has been developed and tested in a CLI model in mice. It has been used to deliver heat shock protein 27 (HSP27) with a transcriptional activator (TAT) derived from the human immunodeficiency virus (HIV) introduced as a protein transduction domain (PTD) to HSP27 along with VEGF.[83] The hypothesis was that the TAT-HSP27 will protect cells in the ischemic environment from apoptosis, while the subsequent release of VEGF induces neovascularization of the ischemic limb. A further layer of complexity was added by the use of either porous or non-porous PLGA spheres. It was found that the combination of TAT-HSP27 and VEGF resulted in a reduction in apoptosis and an increase in arteriole and capillary density, particularly with the porous PLGA spheres. It is worth noting that in the measurement of both apoptosis and angiogenesis, the cumulative effect of TAT-HSP-27 and VEGF was greater than that of either factor alone, underscoring the positive effect of coupling complementary therapeutic strategies.

A fibrin-based sphere-in-gel system has been reported for the delivery of multiple nucleic acids using lipoplexes.[84] This system has proven efficacious in the delivery of *p*eNOS to diabetic rabbit ear ulcer model. However, increased potency of the therapeutic was observed when the secretory control *p*RAB18 was added to the system.[85] The delivery of *p*eNOS and subsequent delivery of *p*RAB18 allowed for increased angiogenesis and reduced inflammation, resulting in improved wound closure at 14 days. Again, delivery of *p*eNOS and *p*RAB18 proved more efficacious than either of the single genes alone, emphasizing the synergistic nature of these genes and strategies. This is depicted in figure 4.

Silk scaffolds have been developed that have dual release, sequential properties. Using either silk nanoparticles[86] or calcium alginate beads,[87] sequential release of factors was achieved. However, in both cases, no therapeutic molecule was added to the system, with model molecules used to assess the release profiles. Also, *in vivo* studies were not performed, making it difficult to comment with any certainty on the potential of the systems.

3 Future Directions and challenges/issues to be overcome

Amongst the systems that have been discussed, a clear distinction should be made. That is between those biomaterial constructs that have potential for clinical translation and those that do not. However, biomaterials that demonstrate multi-modal release have two possible uses: for clinical applications and also for studying interaction between factors. While those suitable for clinical translation may also prove useful in studying biological processes, those which require additional surgical intervention or the application of physical external stimuli are, by contrast, unlikely to be of use in a clinical setting. However, they could prove invaluable as tools to study complex biological processes and how various factors interact during disease/repair/development.

Multi-modal delivery systems have shown much promise in terms of controlling the release of therapeutics and of directing these processes to improve the outcome in a number of disease states. However, even with this promise, there is room for improvement, particularly in the make-up and responsiveness of these systems. An ideal delivery system will be capable of a programmed release to match the requirements of the tissue. Depending on the disease state, and how rapidly the pathology changes, a responsive system may or may not be required. In order to increase the programmability of the scaffold, more carefully constructed biomaterials can be used to exert greater control over the exact make-up and loading regimes of the scaffolds. 3D printing technology has been used to fabricate scaffolds with precise and tunable geometries, and may also be used to exercise precise control over the loading of different factors spatially in a scaffold. Factors may be differentially placed in either the external or internal layers, or in any number of patterns or gradients. In this way, the release can be programmed or fine-tuned to treat different disease states. Jaklenec et al. reported on forming a scaffold by fusing PLGA microspheres using dichloromethane vapor.[88] Using dyes, patterns of bioactivity could be formed in the subsequent scaffolds, paving the way for patterned scaffolds to release multiple factors in a defined yet versatile manner. This scaffold was subsequently used to sequentially release bioactive IGF-1 and TGF-β.[89] This concept of building up scaffolds with different patterns is depicted in figure 2, with three possible patterns shown. This strategy may be particularly suitable for a disease in which the pathology is very well characterized and known.

Multi-modal scaffolds have mostly relied on methods such as differential physical entrapment and degradation to modulate the temporal release of therapeutics, as well as on interactions that delay the release of one factor over another. This is quite crude, and does not fully appreciate the dynamic nature of biological processes of pathology and disease in which the end of one phase often triggers the next. Systems that are more reactive and responsive in nature can prove quite useful in this respect, especially for pathologies with rapid changes between phases. Biomaterial scaffolds have previously incorporated MMP-cleavable linkers[43,44,90,91] to modulate biodegradability. By utilizing this technology, intrinsic properties associated with specific phases of pathology can be used as triggers to release different factors that will allow the biomaterial to dynamically progress with the pathology. For example, MMP, ROS or pH-sensitive linkers can be used to release anti-inflammatory molecules, while low oxygen tension can be used to trigger the release of angiogenic factors in ischemic conditions. Mimicking, or even hijacking, natural processes to engineer responsiveness into biomaterial systems can produce sensitive, flexible and truly reactive systems. This may also help to overcome issues with regard to optimizing the release of the appropriate dose, as the release rate will be related to the intensity of the response, whether it be inflammatory, ischemic or another. Zisch et al. have shown that ‘cell-demanded’ VEGF release (MMP-dependent) results in the formation of a more regular vasculature compared with passive VEGF release via diffusion.[92] This emphasizes the need to involve the host system in the release of any loaded therapeutic. In this way, these systems can be considered both multi-modal as well as responsive in nature.

Antibody technology has been used with varying degrees of success to target systemically administered therapeutics to particular sites. Despite some promising results, much of the therapeutics often end up in the liver, lung and spleen. However, more recently, a slightly different approach has been utilized by combining biomaterial scaffolds and antibodies for tissue engineering. A 'stem cell capturing scaffold' was produced by conjugating the Sca-1 antibody to a collagen scaffold.[93] Sca-1 is a marker for hematopoietic, cardiac and skeletal muscle stem cells, and thus by adding a Sca-1 antibody to the scaffold, these cells would preferentially attach and stay localized to the scaffold. This was shown to increase cardiac regeneration following ischemic injury relative to a collagen scaffold without attached antibody, with increased cell and capillary density. This approach could be used to produce a multimodal scaffold, with defined regions in a scaffold patterned with specific antibodies to promote the attachment of specific cell populations. In addition, specific factors could be loaded into regions of the scaffold to target particular factors to a particular cell type. For instance, a scaffold could preferentially 'capture' macrophages in one layer, and add a factor which will ensure the macrophages are regulatory in nature (IL-10, IL-4) and 'capture' endothelial progenitor cells (EPCs) in another to ensure vascularization. This may also be useful to build up complex layered tissue types *in vivo*, while gradients could potentially be used to regenerate regions such as the osteochondral joint at the knee where the tendon attaches to the bone.

It is clear that multi-modal biomaterial delivery systems offer a new therapeutic modality with which to intervene in many complex and debilitating disease states. However, with this hope come a number of complications that must be considered and overcome. Until now, therapies have focused on the delivery of a single therapeutic, and the effects of that therapeutic. Therapies delivering multiple therapeutics demand an increased understanding of both the mechanism of each of the bioactive molecules as well as of any possible interactions or cross-talk between them. Thus, it becomes clear that the choice of molecules is of paramount importance, both in terms of the efficacy of the therapy and also to ensure no negative interactions that could cause complications for the patient. It is imperative to deliver therapeutics that are complementary to each other in nature, as the combination of therapies should prove more beneficial than either of the therapies alone. If this is not the case, it will be clear that the therapies are not compatible as a dual therapy, and alternative combinations should be pursued. Multi-modal biomaterials are enabling technologies, but they require that the appropriate combination of molecules is carefully selected to ensure maximum benefit from each component. Thus, mechanistic studies to determine interactions between different factors are a prerequisite to combining them for therapeutic intervention.

While multi-modal systems are increasingly being used in a research setting, many difficulties remain in translating these therapies to clinic. One difficulty is to overcome the regulatory barriers for the delivery of two therapeutics. Multiple barriers and restrictions apply when attempting to translate new molecular medicines such as protein, cell and gene therapy to the clinic, even for orphan diseases.[94] Thus, translating a therapy that combines two new therapies is difficult. Therefore, it is easier from a translational standpoint to use multi-modal

biomaterials systems to combine established and approved therapeutics which have already been shown to be efficacious either in the treatment of the disease being targeted or in other disease states. Not only does a therapeutic need to be considered, but also the biomaterial system itself. While increased complexity of the systems is required in terms of the desire to control release, this must be achieved using as simple and as scalable a chemistry as possible, since increased complexity makes it more difficult to translate.[95] Thus, scale-up and issues such as sterilization, packaging and shelf-life are imperative to the successful application of any therapy. In addition, GMP production standards and facilities need to be established before any human trials are possible. In addition to regulatory considerations, practical applications must not be forgotten. The 'device' (biomaterial plus incorporated therapeutics) must be kept simple and should require only minimal manipulation or assembly prior to use to ensure ease of use for the end-user, most likely to be a surgeon treating a patient in an operating room.

4 Summary

Multi-modal biomaterial scaffolds offer promise, not only in terms of the treatment of complex pathologies, but also as a tool to study biological processes and the interaction between different cells and factors. Up to now, multi-modal delivery systems have been programmed for differential release either by using microparticles within a scaffold, or by taking advantage of contrasting affinity of factors with a scaffold. However, in both cases, a lack of true control over release is apparent. For this reason, the next generation of biomaterials will utilize more complex systems that have increased control over release, in terms of reactivity to stimuli as well as the targeting of therapeutics to specific cell types. In this way, the systems attempt to mimic natural processes and move seamlessly from the release of one molecule to another. In spite of the unavoidable complexity in engineering such systems, the ultimate goal must be to ensure that these systems remain amenable to clinical translation.

5 Acknowledgements

The authors would like to acknowledge financial support from Science Foundation Ireland (Grant no. 07/SRC/B1163). The authors would like to thank Mr. Maciej Doczyk for assistance with graphics and Mr. Anthony Sloan for editorial assistance.

6 Notes

[A] Network of Excellence for Functional Biomaterials, National University of Ireland, Galway.

*corresponding author

Email: abhay.pandit@nuigalway.ie; Tel: +353 91 492758; Fax: +353 91 495585

Abbreviations:

ALP – alkaline phosphatase

Ang-1 – angeopoeitin-1

Ang-2 – angeopoeitin-2

bFGF – basic fibroblast growth factor

BMP-2 – bone morphogenic protein 2

BMSC – bone marrow-derived stem cells

BSA – bovine serum albumin

CA4 – combrestatin A4

CAM – chick embryo chorioallantoic membrane

CLI – critical limb ischemia

DX - dexamethasone

ECM – extracellular matrix

EGF – epidermal growth factor

eNOS – endothelial nitric oxide synthase

EPC – endothelial preogenitor cells

EPO - erythropoietin

HGF – hepatocyte growth factor

HSP27 – heat shock protein 27

IGF-1 – insulin-like growth factor

MANHS – methacryloxy n-hydroxysuccinimide

MCP-1 – monocyte chemoattractant protein-1

MI – myocardial infarction

MIP-3α – macrophage inflammatory protein-3 alpha

MMP – matrix metalloproteinases

NIPAAm – n-isopropylacrylamide

NO – nitric oxide

PCL – poly (ε-caprolactone)

PDGF – platelet-derived growth factor

PEG – poly (ethylene glycol)

PEI – poly (ethylenimine)

PELA – poly (DL-lactide) – poly (ethylene glycol)

PLA – poly (lactic acid)

PLG – poly (lactide-co-glycolide)

PLGA - poly (lactide-co-glycolic acid)

PPF – poly (propylene fumarate)

PSA – poly (sebacic acid)

PTD – protein transduction domain

PTX - paclicataxel

RDEB – recessive dystrophic epidermis bullosa

ROS – radical oxygen species

TAT – transcriptional activator

TGF-β – transforming growth factor-beta

TIMPs – tissue inhibitors of matrix metalloproteinases

VEGF – vascular endothelial growth factor

α-SMA – alpha-smooth muscle actin

Table 1: Examples of biomaterial systems that have been used in the literature to deliver multiple factors based on single=phase scaffolds and hydrogels.

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
Alginate gel	VEGF and PDGF	3 μg of each growth factor	Angiogenesis	Release profile – 80% VEGF, 75% PDGF after 30 days 80% weight loss of alginate gel after 40 days	Rat myocardial infarction model	Increased formation of mature blood vessels, improved cardiac function	71
Layered PLG scaffold	VEGF and anti-VEGF	4 μg VEGF in central layer and 20 μg anti-VEGF in surrounding layers	Angiogenesis	Release profile – about 80% of VEGF and anti-VEGF over 30 days	Mouse ischemic hindlimb model	Spatially controlled angiogenesis depending on layers	49
Alginate hydrogel beads	IGF-1 and HGF	170 ng of each factor	Angiogenesis	Release prolife – 100% IGF-1, 25% HGF over 7 days Induction of AKT phosphorylation in cardiac cell cultures Protection of cardiac cell cultures from H_2O_2 induced apoptosis	Rat myocardial infarction model	Increased blood vessel area, reduced apoptosis and reduced fibrotic area	50
Alginate hydrogel	VEGF and IGF-1	3 μg of each factor	Angiogenesis	n/a	Mouse ischemic hindlimb model	Increased blood vessel density, limb reperfusion and tetanic force generation in the anterior tibialis muscles	51
Alginate sulfate hydrogel	VEGF and PDGF-BB, TGF-β	100 ng total growth factor (molar ratio of VEGF: PDGF-BB: TGF- β of 0.6:1:1)	Angiogenesis	TGF-β binding to alginate sulfate Release profile – 45% VEGF, 30% PDGF-BB and TGF-β after 8 days	Subcutaneous rat model	Increased blood vessel area and mature vessels	52
PEG-maleimide hydrogel with growth factor binding and protease- degradable sequences	HGF and VEGF	1 μg of each factor	Angiogenesis	Release profile – 100% release of each factor following treatment with high concentration collagenase, 65% with lower dose, 40% in PBS at 4 days	Rat myocardial infarction model	Increased number of blood vessels, reduced fibrosis and improved myocardial function	53
Brushite-chitosan	PDGF and VEGF	250 ng PDGF and 350 ng VEGF	Bone formation	Release profile – 80% PDGF, 60% VEGF at 21 days	New Zealand rabbit bone defect model	Increased bone formation	54

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
PEG hydrogel (as a coating on single walled carbon nanotubes)	DX and VEGF	0.9 μg DX and 45 ng VEGF released over 9 days	Inflammation and angiogenesis	Release profile - .8 μg DX, 35 ng VEGF at 4 days	Chick embryo chorioallantoic membrane (CAM) assay	Reduced inflammatory cell density and increased blood vessel density	55
2-hydroxyethyl methacrylate, N-vinyl pyrrolidinone, and PEG hydrogel (as a coating on a glucose sensor)	DX and VEGF	20 μg DX and 900 ng VEGF	Inflammation and angiogenesis	n/a	Rat subcutaneous implant	Combination of DX and VEGF reduced inflammation but also reduced angiogenesis	56

Table 2: Examples from the literature of micro- and nanoparticle-based biomaterial systems that have been used to deliver multiple therapeutics.

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
PLA nanocapsules	CA4 and PTX	20 mg/kg CA4 and 5 mg/kg PTX	Anti-angiogenesis and anti-cancer	Nanocapsule size 70nm Release profile – 80% CA4, 60% PTX at 14 days	Mouse primary tumour and liver metastasis model	Reduced tumour volume, reduced angiogenesis and increased survival	57
DOTAP:SPC:Chol:DSPE-PEG2000 cationic liposomes decorated with decorated with low-density lipoprotein receptor-related protein receptor (Angiopep-2) and neuropilin-1 receptor (tLyP-1).	VEGF siRNA and Docetaxel (DX)	1.33 mg/kg VEGF siRNA and 2 mg/kg DX	Anti-angiogenesis and anti-cancer	Uptake of decorated liposomes VEGF expression and cancer cell (U87 MG) survival in response to liposomes/DX	Subcutaneous mouse xenograft tumour model	Reduced tumour weight and volume Reduced tumour VEGF expression Increased apoptosis	58
PEI-PLGA microparticles	IL-10 siRNA and pDNA antigen	50 μg microparticles	Modulation of immune response	Release profile - 90% siRNA at 35 days IL-10 knockdown Increase in expression of CD40, CD86 and 40BBL in primary antigen-presenting cells	Mouse immunization model	Enhanced T-lymphocyte activity and shift towards a Th2 type response	59
Gelatin microspheres	IGF-1 and VEGF	100 μl of 20mg/ml microspheres	Angiogenesis	n/a	Rat myocardial infarction model	Reduced infarct size, increased number of capillaries, reduced inflammation and reduced apoptosis	60
Alginate-albumin particles	FGF-2 and HGF	500ng FGF-2 and 125 ng HGF	Angiogenesis	Proliferation and migration assays to assess dual delivery system	Rat chronic heart failure model	Increased number of blood vessels, reduced collagen density and improved cardiac function	61
PLGA microparticles	VEGF, HGF and Ang-1 (and endothelial progenitor cells)	2.5 μg of each factor	Angiogenesis	n/a	Mouse ischemic hindlimb model	Enhanced neovascularization and perfusion	62
Collagen microspheres	bFGF and HGF	5 μg bFGF and 20 μg HGF	Angiogenesis	n/a	Mouse ischemic hindlimb model	Increased capillary density and maturation index of blood vessels	63

Table 3: Examples from the literature of fibrous scaffolds that have been used to deliver multiple therapeutics.

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
Cellulose acetate hollow fibres	VEGF and S1P	10 µl of 100 µg/ml VEGF or 1800 µM S1P per day	Angiogenesis	Release profile - sustained release of each factor over 24 hours following injection into system	Subcutaneous mouse matrigel plug assay	Increased number of blood vessels and blood vessel maturity	64
Cellulose acetate hollow fibres	bFGF and PDGF	10 µl of 200 ug/ml bFGF or 500 µg/ml PDGF	Angiogenesis	Release profile - sustained release of each factor over 24 hours following injection into system	Subcutaneous mouse matrigel plug assay	Increased number of blood vessels and blood vessel maturity	65
poly(vinyl pyrrolidone)/bovine serum albumin as core fluid and poly(ε-caprolactone) (PCL) solution as sheath fluid	BMSC-affinity peptide and TGF-β1	Not specified	Chondrogenic differentiation	Release profile - burst release over 5 days followed by sustained release up to 21 days BMSC attachment and proliferation Chondrogenic differentiation of BMSCs	n/a	n/a	66
Chitosan hydrogel/poly(ethylene glycol)-b-poly(L-lactide-co-caprolactone) (PELCL) electrospun membrane	VEGF and PDGF	10 µg/ml	Angiogenesis	Release profile – 90% VEGF, 60% PDGF at 15 days Effect of released growth factors on endothelial and smooth muscle cell proliferation	New Zealand white rabbit vascular graft implantation in the left carotid artery	Endothelial cells attached to lumen and smooth muscle cells attached on outer surface, with no thrombosis observed	67
Fibrous PELA scaffold	VEGF and bFGF (pDNA polyplexes)	Not specified	Angiogenesis	Release profile – 40% VEGF, 30% bFGF at 15 days HUVEC attachment to fibrous mats Transfection efficiency VEGF, Collagen IV and Laminin expression	Rat subcutaneous model	Increased blood vessel density	68

Table 4: Examples of sphere-in-scaffold/hydrogel composites that have been used to deliver mutliple therapeutics.

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
PLG scaffold and microspheres	VEGF and PDGF	2 μg VEGF and 3 μg PDGF	Angiogenesis	Release profile – 25% VEGF, less than 10% PDGF after 35 days.	Subcutaneous rat model and mouse ischemic hind limb model	Increased blood vessel density, size and maturity	69
Bilayered PLG scaffold and microspheres	VEGF and PDGF	1.5 μg VEGF and 3 μg VEGF in layer one; 3 μg VEGF in layer two	Angiogenesis	Release profile – 2.5 μg VEGF and 1.5 μg PDGF from layer one over 40 days, and 1 μg VEGF from layer two over 40 days	Mouse ischemic hind limb model	Increased blood vessel density, area and maturity (layer one only)	70
PLG scaffold and microspheres	VEGF, ANG-2 and PDGF, ANG-1	3 μg of each growth factor	Angiogenesis	Endothelial cell sprouting and pericyte detachment assays	Subcutaneous mouse model	Increased blood vessel formation and maturity	39
(NIPAAm)-based hydrogel with PLGA microspheres	bFGF and IGF-1	25 μg/ml bFGF and 1 μg/ml IGF-1 (Total injection volume of 400 μl)	Angiogenesis	Release profile – 50% over 35 days (only measured for bFGF) Bioactivity of released bFGF and IGF-1 (effect on proliferation of rat smooth muscle cells)	Rat myocardial infarction model	No significant differences observed over non-loaded hydrogel	72
Dextran hydrogel and PEI-PLGA microparticles	MIP3α and IL-10 siRNA and pDNA antigen	n/a	Modulation of immune response	Hydrogel swelling ratio MIP3 α release profile – 70-90% release over 3 days (depending on crosslinking regime) Chemokine bioactivity and assessment of chemotaxis IL-10 knockdown in primary antigen-presenting cells	n/a	n/a	73
NIPAAm, HEMA and poly (lactide methacrylate) hydrogel and PLGA spheres	[BSA] as a model protein	n/a	n/a	Complete degradation at 125 days with incorporation of 1 mol% MANHS Shear modulus as a function of temperature Release profile - 100% release from hydrogel at 100 days, 50% release from PLGA spheres at 200 days	n/a	n/a	74

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
Chitosan gel and gelatin microspheres	BMP-2 and IGF-1	n/a	Osteoblastic differentiation	Swelling, cytotoxicity and degradation of gelatin microspheres Release profile – 35 ng/ml BMP-2 and 15 ng/ml IGF-1 at 7 days Increased alkaline phosphate activity	n/a	n/a	75
Poly (propylene fumarate) (PPF) and gelatin microparticles	VEGF and BMP-2	12 μg VEGF and 2 μgBMP-2	Bone formation	n/a	Rat critical-sized bone defect	Increased bone volume	76
PPF and gelatin microparticles	VEGF and BMP-2 [dose study]	VEGF dose of 0, 6 or 12 μg and BMP-2 dose of 0, 6 or 12 μg	Bone formation	Release profile – 90% VEGF released at 5 days, 40% BMP-2 released at 25 days (both in collagenase buffer)	Rat critical-sized bone defect	Decreased bone formation as BMP-2 dose was reduced, minimal effect of VEGF dose	77
PLGA microspheres and PPF scaffold surrounded by a gelatin hydrogel	VEGF and BMP-2	2 μg VEGF and 9.2 μg BMP-2	Bone formation	Release profile – 90% VEGF, 20% BMP-2 at 7 days	Rat subcutaneous and critical sized bone defect	Increased blood vessel volume and bone volume (subcutaneous model)	78
Collagen/fibronectin hydrogel and alginate microparticles	VEGF and MCP-1	1000 ng/mg VEGF and 50 ng/mg MCP-1	Angiogenesis	Release profile – 100% VEGF, 75% MCP-1 at 2 days	Subcutaneous mouse model	Increased number of blood vessels, blood vessel diameter and blood vessel maturity	79
PLGA and PLGA/PSA microspheres in a HA/methycellulose hydrogel	EGF and EPO	Not specified	Brain regeneration	Release profile - 80% EGF, 25% EPO at 10 days	Mouse stroke model	Reduced inflammation, apoptosis and cell death, increased neuronal repair	80
Alginate gel and PLGA microspheres	HSP27 and VEGF	3 μg TAT HSP-27 and 0.65 μg VEGF	Anti-apoptosis and angiogenesis	100% HSP27, 30% VEGF at 10 days	Mouse ischemic hindlimb model	Reduced apoptosis, increased arteriole and capillary density	83
PLG scaffolds and microspheres	VEGF and FGF-2	4 μg VEGF and 2 μg FGF-2	Angiogenesis	Protein encapsulation efficiency and protein remaining after scaffold leaching Release profile – dependent on loading method Bioactivity of released proteins confirmed	Rat spinal cord hemisection model	Increased endothelial cell infiltration	48

Biomaterial system	Therapeutics	Doses used	Target	In vitro characterisation	In vivo model	Effect observed	Reference
Fibrin gel and microspheres	eNOS and RAB18 (pDNA lipoplexes)	10 μg of each pDNA	Angiogenesis and inflammation	[Release profile previously characterized]	Diabetic rabbit ear ulcer model	Reduced inflammation, increased angiogenesis and improved wound closure	85

7 References

1. J. K. Tessmar and A. M. Göpferich, *Adv. Drug Delivery Rev.*, 2007, **59**, 274–91.
2. L. De Laporte and L. D. Shea, *Adv. Drug Delivery Rev.*, 2007, **59**, 292–307.
3. J. M. Dang and K. W. Leong, *Adv. Drug Delivery Rev.*, 2006, **58**, 487–99.
4. J. D. Kretlow, L. Klouda, and A. G. Mikos, *Adv. Drug Delivery Rev.*, 2007, **59**, 263–73.
5. A. C. A. Wan and J. Y. Ying, *Adv. Drug Delivery Rev.*, 2010, **62**, 731–40.
6. M. P. Lutolf and J. A. Hubbell, *Nat. Biotechnol.*, 2005, **23**, 47–55.
7. E. S. Place, N. D. Evans, and M. M. Stevens, *Nat. Mater.*, 2009, **8**, 457–70.
8. C. Holladay, M. Keeney, U. Greiser, M. Murphy, T. O'Brien, and A. Pandit, *J. Controlled Release*, 2009, **136**, 220–5.
9. M. Keeney, J. J. J. P. van den Beucken, P. M. van der Kraan, J. A. Jansen, and A. Pandit, *Biomaterials*, 2010, **31**, 2893–902.
10. R. Visser, P. M. Arrabal, J. Becerra, U. Rinas, and M. Cifuentes, *Biomaterials*, 2009, **30**, 2032–7.
11. L. Hong, I. Peptan, P. Clark, and J. J. Mao, *Ann. Biomed. Eng.*, 2005, **33**, 511–517.
12. Y. Lei, M. Rahim, Q. Ng, and T. Segura, *J. Controlled Release,* 2011, **153**, 255–61.
13. H. J. Kong, E. S. Kim, Y.-C. Huang, and D. J. Mooney, *Pharm. Res.*, 2008, **25**, 1230–8.
14. J. C. Garbern, E. Minami, P. S. Stayton, and C. E. Murry, *Biomaterials*, 2011, **32**, 2407–16.
15. C. M. Kirschner and K. S. Anseth, *Acta Mater.*, 2013, **61**, 931–944.
16. B. V Slaughter, S. S. Khurshid, O. Z. Fisher, A. Khademhosseini, and N. A. Peppas, *Adv Mater.*, 2009, **21**, 3307–29.
17. S. J. Kew, J. H. Gwynne, D. Enea, R. Brookes, N. Rushton, S. M. Best, and R. E. Cameron, *Acta Biomater.*, 2012, **8**, 3723–31.
18. W. T. Daly, L. Yao, M. T. Abu-Rub, C. O'Connell, D. I. Zeugolis, A. J. Windebank, and A. S. Pandit, *Biomaterials*, 2012, **33**, 6660–71.
19. S. Browne, G. Fontana, B. J. Rodriguez, and A. Pandit, *Mol. Pharmaceutics*, 2012, **9**, 3099–106.
20. B. C. Dash, G. Réthoré, M. Monaghan, K. Fitzgerald, W. Gallagher, and A. Pandit, *Biomaterials*, 2010, **31**, 8188–8197.
21. B. C. Dash, S. Mahor, O. Carroll, A. Mathew, W. Wang, K. a Woodhouse, and A. Pandit, *J. Controlled Release*, 2011, **152**, 382–92.
22. Z. S. Patel, H. Ueda, M. Yamamoto, Y. Tabata, and A. G. Mikos, *Pharm. Res.*, 2008, **25**, 2370–8.

23. J. C. Sy, G. Seshadri, S. C. Yang, M. Brown, T. Oh, S. Dikalov, N. Murthy, and M. E. Davis, *Nat. Mater.*, 2008, **7**, 863–8.

24. O. C. M. Chan, K.-F. So, and B. P. Chan, *J. Controlled Release*, 2008, **129**, 135–43.

25. J. C. Sy, E. a Phelps, A. J. García, N. Murthy, and M. E. Davis, *Biomaterials*, 2010, **31**, 4987–94.

26. S. Franz, S. Rammelt, D. Scharnweber, and J. C. Simon, *Biomaterials*, 2011, **32**, 6692–6709.

27. J. M. Anderson, A. Rodriguez, and D. T. Chang, *Semin. Immunol.*, 2008, **20**, 86–100.

28. Z. Xia and J. T. Triffitt, *Biomed. Mater.*, 2006, **1**, R1–9.

29. W. Kenneth Ward, *J. Diabetes Sci. Tech.*, 2008, **2**, 768–77.

30. S. M. van Putten, D. T. A. Ploeger, E. R. Popa, and R. A. Bank, *Acta Biomater.*, 2013, **9**, 6502–10.

31. E. A. Phelps and A. J. García, *Curr. Opin. Biotechnol.*, 2010, **21**, 704–9.

32. A. Järvikallio, L. Pulkkinen, and J. Uitto, *Human Mutation*, 1997, **347**, 338–47.

33. J. Uitto and A. M. Christiano, *Arch. Dermatol. Res.*, 1994, **287**, 16–22.

34. S. Enoch, *Surgery*, 2005, **23**, 37–42.

35. T. J. Koh and L. A. DiPietro, *Expert Rev. Mol. Med.*, 2011, **13**, 1–12.

36. G. C. Gurtner, S. Werner, Y. Barrandon, and M. T. Longaker, *Nature*, 2008, **453**, 314–21.

37. S. A. Eming, T. Krieg, and J. M. Davidson, *J. Invest. Dermatol.*, 2007, **127**, 514–25.

38. K. Kinnunen, P. Korpisalo, T. T. Rissanen, T. Heikura, H. Viita, H. Uusitalo, and S. Ylä-Herttuala, *Acta Physiol.*, 2006, **187**, 447–57.

39. Y. Brudno, A. B. Ennett-Shepard, R. R. Chen, M. Aizenberg, and D. J. Mooney, *Biomaterials*, 2013, **34**, 9201–9.

40. V. Falanga, *Lancet*, 2005, **366**, 1736–43.

41. A. Breen, G. Mc Redmond, P. Dockery, T. O'Brien, and A. Pandit, *J. Invest. Surg.*, 2008, **21**, 261–9.

42. M. R. Schäffer, U. Tantry, P. A. Efron, G. M. Ahrendt, F. J. Thornton, and A. Barbul, *Surgery*, 1997, **121**, 513–519.

43. M. P. Lutolf, J. L. Lauer-Fields, H. G. Schmoekel, A. T. Metters, F. E. Weber, G. B. Fields, and J. A. Hubbell, *Proc. Natl. Acad. Sci. U.S.A.*, 2003, **100**, 5413–8.

44. J. Patterson and J. A. Hubbell, *Biomaterials*, 2010, **31**, 7836–45.

45. S. E. Sakiyama-Elbert and J. A. Hubbell, *J. Controlled Release*, 2000, **69**, 149–58.

46. S. E. Sakiyama-Elbert and J. A. Hubbell, *J. Controlled Release*, 2000, **65**, 389–402.

47. A. H. Zisch, U. Schenk, J. C. Schense, S. E. Sakiyama-Elbert, and J. A. Hubbell, *J. Controlled Release*, 2001, **72**, 101–13.

48. L. De Laporte, A. des Rieux, H. M. Tuinstra, M. L. Zelivyanskaya, N. M. De Clerck, A. A. Postnov, V. Préat, and L. D. Shea, *J. Biomed. Mater. Res. Part A*, 2011, **98**, 372–82.

49. W. W. Yuen, N. R. Du, C. H. Chan, E. A. Silva, and D. J. Mooney, *Proc. Natl. Acad. Sci. U.S.A.*, 2010, **107**, 17933–8.

50. E. Ruvinov, J. Leor, and S. Cohen, *Biomaterials*, 2011, **32**, 565–78.

51. C. Borselli, C. A. Cezar, D. Shvartsman, H. H. Vandenburgh, and D. J. Mooney, *Biomaterials*, 2011, **32**, 8905–14.

52. I. Freeman and S. Cohen, *Biomaterials*, 2009, **30**, 2122–31.

53. A. S. Salimath, E. A. Phelps, A. V Boopathy, P.-L. Che, M. Brown, A. J. García, and M. E. Davis, *PloS one*, 2012, **7**, e50980.

54. B. De la Riva, E. Sánchez, A. Hernández, R. Reyes, F. Tamimi, E. López-Cabarcos, A. Delgado, and C. Evora, *Journal of Controlled Release*, 2010, **143**, 45–52.

55. J. Sung, P. W. Barone, H. Kong, and M. S. Strano, *Biomaterials*, 2009, **30**, 622–31.

56. L. W. Norton, H. E. Koschwanez, N. A. Wisniewski, B. Klitzman, and W. M. Reichert, *J. Biomed. Mater. Res. Part A*, 2007, **81**, 858–69.

57. Z. Wang and P. C. Ho, *Biomaterials*, 2010, **31**, 7115–23.

58. Z.-Z. Yang, J.-Q. Li, Z.-Z. Wang, D.-W. Dong, and X.-R. Qi, *Biomaterials*, 2014, **In press**.

59. A. Singh, H. Nie, B. Ghosn, H. Qin, L. W. Kwak, and K. Roy, *Mol. Ther.*, 2008, **16**, 2011–21.

60. A. Cittadini, M. G. Monti, V. Petrillo, G. Esposito, G. Imparato, A. Luciani, F. Urciuolo, E. Bobbio, C. F. Natale, L. Saccà, and P. A. Netti, *Eur. J. Heart Fail.*, 2011, **13**, 1264–74.

61. S. Banquet, E. Gomez, L. Nicol, F. Edwards-Lévy, J.-P. Henry, R. Cao, D. Schapman, B. Dautreaux, F. Lallemand, F. Bauer, Y. Cao, C. Thuillez, P. Mulder, V. Richard, and E. Brakenhielm, *Circulation*, 2011, **124**, 1059–69.

62. J. Saif, T. M. Schwarz, D. Y. S. Chau, J. Henstock, P. Sami, S. F. Leicht, P. C. Hermann, S. Alcala, F. Mulero, K. M. Shakesheff, C. Heeschen, and A. Aicher, *Arterioscler. Throm. Vasc. Biol*, 2010, **30**, 1897–904.

63. A. Marui, A. Kanematsu, K. Yamahara, K. Doi, T. Kushibiki, M. Yamamoto, H. Itoh, T. Ikeda, Y. Tabata, and M. Komeda, *J. Vasc. Surg.*, 2005, **41**, 82–90.

64. J. E. Tengood, K. M. Kovach, P. E. Vescovi, A. J. Russell, and S. R. Little, *Biomaterials*, 2010, **31**, 7805–12.

65. J. E. Tengood, R. Ridenour, R. Brodsky, A. J. Russell, and S. R. Little, *Tissue Eng. Part A*, 2011, **17**, 1181–9.

66. Z. Man, L. Yin, Z. Shao, X. Zhang, X. Hu, J. Zhu, L. Dai, H. Huang, L. Yuan, C. Zhou, H. Chen, and Y. Ao, *Biomaterials*, 2014, **In press**.

67. H. Zhang, X. Jia, F. Han, J. Zhao, Y. Zhao, Y. Fan, and X. Yuan, *Biomaterials*, 2013, **34**, 2202–12.

68. S. He, T. Xia, H. Wang, L. Wei, X. Luo, and X. Li, *Acta Biomater*, 2012, **8**, 2659–69.

69. T. P. Richardson, M. C. Peters, A. B. Ennett, and D. J. Mooney, *Nat. Biotechnol.*, 2001, **19**, 1029–1034.

70. R. R. Chen, E. A. Silva, W. W. Yuen, and D. J. Mooney, *Pharm. Res.*, 2007, **24**, 258–64.

71. X. Hao, E. A. Silva, A. Månsson-Broberg, K.-H. Grinnemo, A. J. Siddiqui, G. Dellgren, E. Wärdell, L. A. Brodin, D. J. Mooney, and C. Sylvén, *Cardiovasc. Res.*, 2007, **75**, 178–85.

72. D. M. Nelson, R. Hashizume, T. Yoshizumi, A. K. Blakney, Z. Ma, and W. R. Wagner, *Biomacromolecules*, 2014, **15**, 1–11.

73. A. Singh, S. Suri, and K. Roy, *Biomaterials*, 2009, **30**, 5187–200.

74. D. M. Nelson, Z. Ma, C. E. Leeson, and W. R. Wagner, *J. Biomed. Mater. Res. Part A*, 2012, **100**, 776–85.

75. S. Kim, Y. Kang, C. A. Krueger, M. Sen, J. B. Holcomb, D. Chen, J. C. Wenke, and Y. Yang, *Acta Biomater.*, 2012, **8**, 1768–77.

76. Z. S. Patel, S. Young, Y. Tabata, J. A. Jansen, M. E. K. Wong, and A. G. Mikos, *Bone*, 2008, **43**, 931–40.

77. S. Young, Z. S. Patel, J. D. Kretlow, M. B. Murphy, P. M. Mountziaris, L. S. Baggett, H. Ueda, Y. Tabata, M. Wong, J. A. Jansen, and A. G. Mikos, *Tissue Eng. Part A*, 2009, **15**, 2347–62.

78. D. H. R. Kempen, L. Lu, A. Heijink, T. E. Hefferan, L. B. Creemers, A. Maran, M. J. Yaszemski, and W. J. A. Dhert, *Biomaterials*, 2009, **30**, 2816–25.

79. S. M. Jay, B. R. Shepherd, J. W. Andrejecsk, T. R. Kyriakides, J. S. Pober, and W. M. Saltzman, *Biomaterials*, 2010, **31**, 3054–62.

80. Y. Wang, M. J. Cooke, N. Sachewsky, C. M. Morshead, and M. S. Shoichet, *J. Controlled Release*, 2013, **172**, 1–11.

81. S. Hirabara, T. Kojima, N. Takahashi, M. Hanabayashi, and N. Ishiguro, *Biochem. Biophy. Res. Com.*, 2013, **430**, 519–522.

82. K. Nakamura, S. Yokohama, M. Yoneda, S. Okamoto, Y. Tamaki, T. Ito, M. Okada, K. Aso, and I. Makino, *J. Gastroenterology*, 2004, **39**, 346–354.

83. S.-H. Shin, J. Lee, K. S. Lim, T. Rhim, S. K. Lee, Y.-H. Kim, and K. Y. Lee, *J. Controlled Release*, 2013, **166**, 38–45.

84. M. M. Kulkarni, U. Greiser, T. O'Brien, and A. Pandit, *Mol. Pharmaceutics*, 2011, **8**, 439–46.

85. M. Kulkarni, A. O. Loughlin, R. Vazquez, K. Mashayekhi, P. Rooney, U. Greiser, E. O. Toole, T. O. Brien, M. M. Malagon, and A. Pandit, *Biomaterials*, 2014, **35**, 2001–10.

86. K. Numata, S. Yamazaki, and N. Naga, *Biomacromolecules*, 2012, **13**, 1383–9.

87. B. B. Mandal and S. C. Kundu, *Biomaterials*, 2009, **30**, 5170–7.

88. A. Jaklenec, E. Wan, M. E. Murray, and E. Mathiowitz, *Biomaterials*, 2008, **29**, 185–92.

89. A. Jaklenec, A. Hinckfuss, B. Bilgen, D. M. Ciombor, R. Aaron, and E. Mathiowitz, *Biomaterials*, 2008, **29**, 1518–25.

90. S. T. Wall, C.-C. Yeh, R. Y. K. Tu, M. J. Mann, and K. E. Healy, *J. Biomed. Mater. Res. Part A*, 2010, **95**, 1055–66.

91. J. Su, M. Eng, S. T. Wall, D. Ph, K. E. Healy, and C. F. Wildsoet, *Tissue Eng. Part A*, 2010, **16**, 905–16.

92. A. H. Zisch, M. P. Lutolf, and J. A. Hubbell, *Cardiovascular Pathology*, 2003, **12**, 295–310.

93. C. Shi, Q. Li, Y. Zhao, W. Chen, B. Chen, Z. Xiao, H. Lin, L. Nie, D. Wang, and J. Dai, *Biomaterials*, 2011, **32**, 2508–15.

94. M. P. Messenger and P. E. Tomlins, *Adv. Health. Mater.*, 2011, **23**, 10–7.

95. G. D. Prestwich, S. Bhatia, C. K. Breuer, L. Shannon, M. Dahl, C. Mason, R. Mcfarland, D. J. Mcquillan, J. Schox, W. E. Tente, and A. Trounson, *Sci. Trans. Med.*, 2012, **4**, 1–6.

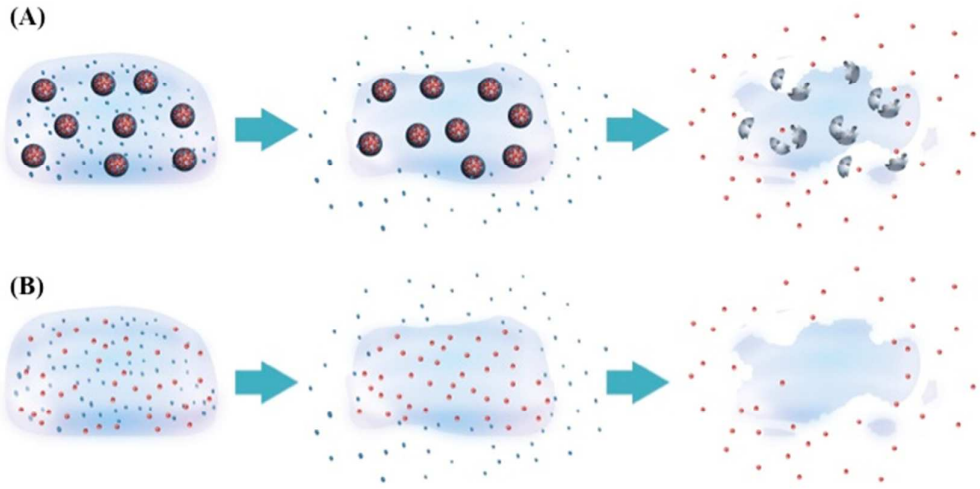

Figure 1: Two typical strategies that have been used to achieve multi-modal release. (A) The use of microspheres in a scaffold to promote differential release of two factors, as the contents of the spheres (drawn in red) are released slower than the contents of the hydrogel (drawn in blue). (B) The use of materials that have a differential affinity for the biomaterial. In this case, the factor drawn in red is released slower than the factor drawn in blue. This may be a natural phenomenon due to the interaction between the biomaterial and the loaded therapeutics, or may be engineered into the material using linker systems.

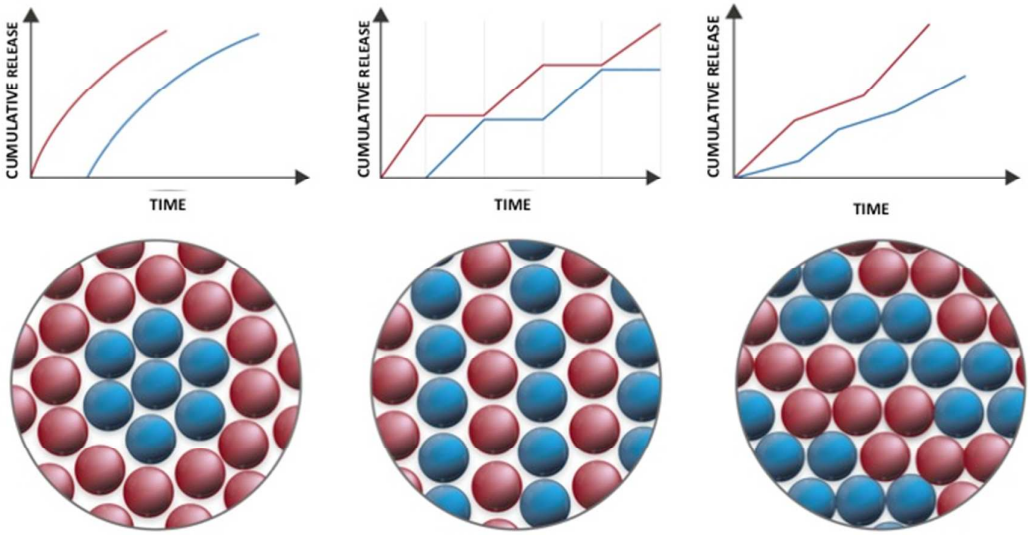

Figure 2: Possible strategies to create multi –modal biomaterial delivery systems. Patterns of spheres (containing different factors loaded in either blue or red spheres) may be built up to create systems with tailored release profiles. Three possible patterns, and their potential representative release profiles, are shown. The red curve represents the release of therapeutics from red spheres shown in the diagram while the blue curve represents the release of therapeutics from blue spheres.

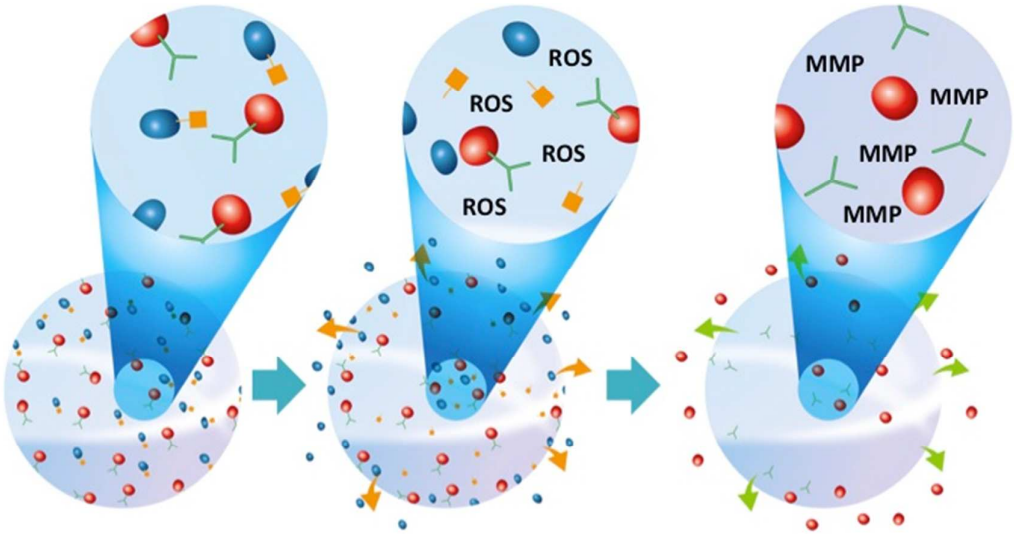

Figure 3: A mechanism to achieve responsive multi-modal release. In this case two separate linkers (depicted in green and orange) are used to bind two different factors (depicted in blue and red) to the scaffold, resulting in differential, responsive release related to the local microenvironment. In this case, the orange linkers are radical oxygen species (ROS) sensitive while the green linkers are matrix metalloproteinase (MMP) sensitive as shown in the zoomed in views. The linkers may be responsive to a number of phenomenon such as ROS and MMPs as shown here as well as pH or hypoxia etc., and in this way can respond to the pathophysiology.

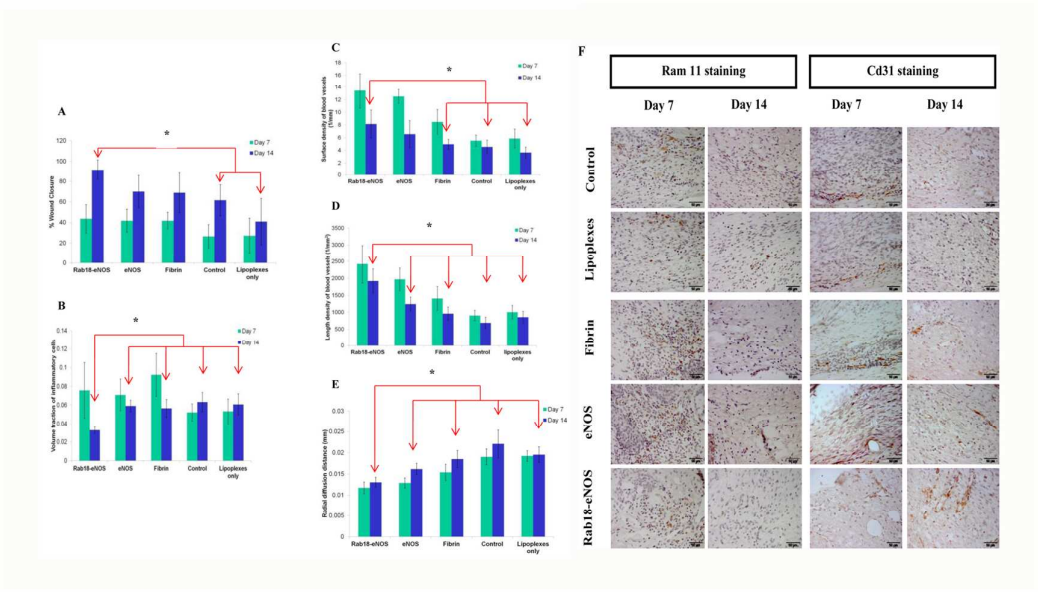

Figure 4: Multi-modal delivery of eNOS and RAB-18 results in (A) improved wound closure. Combined delivery of eNOS and RAB-18 results in (B) reduced volume fraction of inflammatory cells, (C) increased length density and (D) surface density of blood vessels and (E) a reduced radial diffusion distance. Ram 11 and CD31 staining confirmed reduced macrophage presence and an increased number of blood vessels (F). Reprinted from Biomaterials, 35, M. Kulkarni, A. O. Loughlin, R. Vazquez, K. Mashayekhi, P. Rooney, U. Greiser, E. O. Toole, T. O. Brien, M. M. Malagon, and A. Pandit, Use of a fibrin-based system for enhancing angiogenesis and modulating inflammation in the treatment of hyperglycemic wounds, pages 2001-2010, copyright 2014, with permission from Elsevier (85).