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Incorporation of Heparin into Biomaterials

Shelly E Sakiyama-Elbert, PhD
Department of Biomedical Engineering, Washington University, 1 Brookings Drive, Campus Box 1097, St. Louis, MO 63130 USA

Shelly E Sakiyama-Elbert: Sakiyama@wustl.edu

Abstract

This review provides an overview on the incorporation of heparin into biomaterials with a focus on drug delivery and the use of heparin-based biomaterials for self-assembly of polymer networks. Heparin conjugation to biomaterials was originally explored to reduce the thrombogenicity of materials in contact with blood. Many of the conjugation strategies that were developed for these applications are still popular today for other applications. More recently heparin has been conjugated to biomaterials for drug delivery applications. Many of the delivery approaches have taken advantage of the ability of heparin to bind to a wide variety of growth factors, protect them from degradation and to potentiate their interactions with cell surface receptors. More recently, the use of heparin as a base polymer for scaffold fabrication has also been explored, often utilizing non-covalent binding of heparin with peptides or proteins to promote self-assembly of hydrogel networks. This review will highlight recent advances in each of these areas.

INTRODUCTION

Heparin and heparin sulfate are linear polysaccharides. Both are synthesized from a common precursor proteoglycan. Heparin is only produced in mast cells, where it is cleaved from the core protein (serglycin) at the end of the synthesis [1]. Heparin sulfate (HS) is found in most tissues and remains attached to the core protein. Both are sulfated and also contain carboxylic acids, which contribute to an overall net negative charge [2]. Heparin/HS polymer chains are made up of repeating disaccharides, primarily uronic acid and glucosamine with varying degrees of sulfation and Nacetylation (Figure 1). While their interactions with proteins are largely electrostatic, there are clearly contributions from hydrophobic effects and hydrogen bonding, as well as promoting secondary structure in the proteins binding to heparin, which imparts some selectivity and specificity [3]. In addition to binding to growth factors, heparin also binds to a number of enzymes (e.g. antithrombin III), plasma proteins (platelet factor 4), and extracellular matrix (ECM) proteins (e.g. fibronectin, laminin) [4, 5]. In some cases, specific heparin sulfation codes have been identified that facilitate binding with growth factors (e.g. bFGF) or enzymes (e.g. antithrombin III) [6, 7].

Disclosure of Conflict of Interest:
SSE is an inventor on patents regarding the use of heparin binding drug delivery and these patents are licensed to Kuros Therapeutics. SSE may receive royalties from Kuros from sub-licensing revenue. SSE is not in any way involved in consulting or operations at Kuros.

Heparin Modification of Materials to Reduce Thrombogenecity

Heparin was discovered in 1916 and has been used as an anticoagulant clinically since 1935 [1]. Modification of biomaterials with heparin has been performed for over 50 years. Initially, heparin was immobilized via ionic interactions to reduce the thrombogenecity of materials in the 1960's [8, 9]. This approach took advantage of electrostatic interactions with the negatively charge sulfate groups on heparin with the colloidal graphite and benzalkonium chloride (cation) in alternating layers [8]. Leininger *et al.* adapted this method for use on plastic surfaces by forming quaternary ammonium sites on the material surface to promote electrostatic interactions with the heparin [10].

In the 80's, methods for covalent conjugation were developed that used end-point immobilization, in which a primary amine on the material of interest was reacted with an aldehyde group generated by heparin chain depolymerization [11]. The literature on heparin immobilization is vast and has been reviewed extensively elsewhere [12–15]. This end-point immobilization has been use to conjugate heparin to vascular grafts and has been commercialized for ePTFE and Dacron grafts [16, 17]. More recently work with heparin immobilization on vascular grafts has explored mechanisms other than antithrombotic effects that may be influence by heparin, including elastin synthesis [18]. Additional studies explored coating vascular stents with heparin, however more recent studies suggest that this may stimulate restenosis by sequestration of growth factors that promote smooth muscle cell proliferation [19].

HEPARIN MODIFICATION OF MATERIALS FOR DRUG DELIVERY

Many types of drug delivery systems have been developed for the control release of small molecule and protein-based drugs for biomedical applications [20]. For delivery of protein-based drugs, such as growth factors, there are many advantages to the use of affinity drug delivery systems, such as heparin-based delivery systems. These affinity delivery systems utilize specific non-covalent interactions to stabilize drugs and immobilize them within a biomaterial matrix, thus protecting their biological activity and slowing their diffusion from the matrix. The interactions with growth factors and affinity delivery systems can mimic those that naturally occur with native ECM proteoglycans.

Because a large number of growth factors bind to heparin with either moderate or high affinity (~10^{-6} – 10^{-9} M K_D), heparin-based delivery systems have proven useful for the delivery of a wide range for growth factors for different biomedical applications [21]. In the case of heparin-based systems, these interactions can also modulate the binding of growth factor to the cell surface receptor. For some growth factors, such as basic fibroblast growth factor (bFGF), heparin facilitates this binding of bFGF to its receptor and actually increases activity [22]. However for other growth factors, such as bone morphogenetic protein 2 (BMP2), heparin can inhibit binding to the cell surface receptor [23]. The effect of heparin on BMP2 signaling are complex, as heparin has also been shown to block inhibition by noggin of the BMP2 pathway [24], thus demonstrating that heparin can have direct interactions with the growth factors and their receptors, as well as indirect and sometimes opposing effects on signaling cascades.

Growth factors that bind to heparin include commonly studied heparin-binding growth factors, such as bFGF and vascular endothelial growth factor (VEGF), as well as members of transforming growth factor (TGF, e.g. BMPs), platelet derived growth factor (PDGF), epidermal growth factor (EGF), and hepatocyte growth factor families [25–28]. Other morphogens, such as sonic hedgehog (Shh) and pathogens, such as *B. pertussis*, *herpes simplex virus (HSV)* and *Plasmodium falciparum*, also bind to heparin and can be delivered or sequestered using a similar approach [29, 30].

Covalent conjugation of heparin to biomaterials for delivery

Early work in demonstrating the utility of heparin-based delivery was performed by Edelman and Langer. Their initial system utilized heparin-conjugated Sepharose beads to bind bFGF within alginate microspheres. Their preliminary studies *in vitro* demonstrated that active bFGF could be released for at least two weeks and heparin enhanced growth factor activity [31]. They went on to demonstrate the delivery of bFGF from heparin-Sepharose bead in alginate stimulated angiogenesis and neointimal proliferation in a rat carotid artery model [32]. Later, a Phase I clinical trial using these materials showed that bFGF improved revascularization after coronary artery bypass in a small number of patients [33]. Additionally, a Phase II trial demonstrated improved revascularization with bFGF treatment and a trend toward increased left ventricular ejection fraction [34]. These studies demonstrate that heparin-based delivery can be used to provide sustained release of growth factors in a clinical model.

Another approach to covalently immobilize heparin to biomaterials was to covalently link it to a protein, such as collagen or albumin, using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) [35]. Heparin was conjugated to albumin using this method and emulsified to form microsphere that could then be covalently crosslinked with glutaraldehyde [36]. A similar approach was used crosslink heparin to collagen matrices for the delivery of bFGF, and bFGF delivery was found to enhance endothelial cell proliferation *in vitro* [37, 38]. Later *in vivo* studies demonstrated that bFGF delivery from similar collagen matrices increased vascularization for three weeks in a rat subcutaneous implant model [39, 40].

Similar EDC chemistry can be use to immobilize heparin onto poly(L-lactide-co-glycolide) (PLGA)-based materials. Jeon *et al.* developed heparin-conjugated PLGA nanospheres by first reacting PLGA with t-Boc protected-glycine and then deprotecting. Nanospheres of PLGA were formed using an oil/water emulsion and reacted with heparin in the presence of EDC/N-hydroxysuccinimide (NHS) [41]. Controlled release of bFGF and increased cell proliferation was observed over 28 days *in vitro*. They observed increased capillary density with bFGF and PLGA nanospheres versus controls in a mouse ischemic limb model. Similar heparin-conjugation methods have also been used with salt-leached PLGA scaffolds to deliver BMP2 and BMP2 delivery increased bone formation in an ectopic bone formation model compared to PLGA scaffolds without heparin [42].

Coupling via end point reductive amination has also been used to covalently attach heparin to various biomaterials (similar to strategy most commonly used for used for vascular graft coupling). Hyaluronic acid (HA) was modified to contain amine groups by Liu *et al.* (using periodate and then amine groups were added using ethylene diamine in the presence of cyanoborohydride). Aminated-HA was then reacted this with heparin-aldehyde via reductive amination [43]. Controlled release of bFGF from HA scaffolds via enzymatic degradation of the HA and biological activity of the released bFGF (cell proliferation) was observed in these studies. More recently novel methods for site selective aldehyde modification have been developed that allow modification at specific sites on heparin [44].

Modification of heparin to contain thiol groups is another method for adding an additional reactive functionality to heparin that can reacts with many existing polymer end groups (e.g. acrylates, vinyl sulfones). A method for thiolation of glycosaminoglycans (GAGs), such as HA and heparin was developed by the Prestwich lab [45] that utilizes EDC and dithiothreitol (DTT). The thiolated GAGs (HA and heparin) are then reacted with PEG diacrylate via Michael type addition to generate PEG-HA-heparin hydrogels [46]. The release of growth factor from these gels is dependent on hyaluronidase for degradation of gels containing HA (as part of the GAG component) and bFGF release was sustained for at least 28 days *in*

vitro. Delivery of bFGF from these scaffolds was also found to promote neovascularization *in vivo* in a mouse subcutaneous implant model.

This approach for use with PEG diacrylate hydrogels in the absence of HA by Tae *et al.*, and they demonstrated that fibroblasts could be encapsulated within these gels [47]. Delivery of human growth hormone (hGH) from hydrogels formed from thiolated heparin and PEG diacrylate by Michael type addition, rather than by photo-polymerization has also been explored [48]. These hydrogels were shown to promote increased vascularization in a subcutaneous mouse implant model when osteoprotegerin, a pro-angiogenic factor from the tumor necrosis factor (TNF) superfamily, was delivered using this method *in vivo* [49]. This thiolation method also shows great potential for a broad variety of applications, and the thiol chemistry is somewhat more selective due to the relatively low incidence (1–4% frequency) of thiols (cysteines) in protein sequences compared to amine groups [50].

Maleimide groups can also provide another unique functionality to react with polymer end groups for conjugation of heparin to materials. Heparin-containing PEG hydrogels can be made by reacting PEG-tetrathiol with low molecular weight heparin-maleimide. The PEG-heparin conjugates are then reacted with PEG-tetraHIP (heparin interacting peptide) [51] to form gels, and these gels can sequester bFGF. This approach can also be modified to react PEG-dithiol with high molecular weight heparin-maleimide and controlled bFGF can be obtained from these gels as well [52]. This approach can be modified to work well with the thiol-based approaches described above.

Heparin can also be modified by the addition of hydrazide groups to carboxylic acids on heparin for covalent attachment to polymers using a method develop by Bulpitt and Aeschlimann [53]. Tae *et al.* used this method to reacted hydrazide modified heparin with NHS ester of PEG-bis-butanoic acid (SBA-PEG-SBA) to form hydrogels. They demonstrated sustained VEGF release *in vit*ro and increased angiogenesis *in vivo* in a subcutaneous mouse implant model [54].

Non-covalent immobilization of heparin for delivery

A novel method for immobilization of heparin within biomaterials was developed by Sakiyama-Elbert and Hubbell, using non-covalent (primarily electrostatic) interactions rather than covalent immobilization [3]. The three-component delivery system consisted of a heparin-binding peptide covalently immobilized to the biomaterial, heparin and a heparin-binding growth factor. To demonstrate the feasibility of this approach, a peptide containing a modified version of the heparin-binding domain of antithrombin III was crosslinked into fibrin matrices using the transglutaminase activity of Factor XIIIa. They demonstrated that bFGF could be released in a controlled manner from this delivery system [55]. They also demonstrated that this approach can also be used with growth factors that possess only moderate heparin binding affinity ($K_D \sim 10^{-6}$) via short basic domains that are accessible in protein surfaces, such as nerve growth factor (NGF), neurotrophin 3 (NT-3) and brain derived neurotrophic factor (BDNF) [56].

Others have shown that this approach can be expanded to other materials, such as polyethylene glycol (PEG) hydrogels, for the delivery of heparin-binding growth factors. Pratt *et al.* demonstrated that BMP2 can be delivered from plasmin-degradable PEG hydrogels via a heparin-binding peptide/heparin complex and promotes improved bone healing in a rat cranial defect model [57]. This approach has also been used for delivery of PDGF-BB to enhance gliding after mid-substance injury in a canine flexor tendon model [58–60].

The effect of binding site affinity on the rate of release has also been explored. To identify peptide sequences with a broader range of affinities for heparin, a phage display library was screened to identify sequences with varying affinity for heparin [61]. The role of heparin-binding affinity for heparin was evaluated for electrostatic immobilization of heparin, and increasing heparin-binding affinity has been shown to provide a longer duration of release [62]. Recently, Wieduwild *et al.* explored the effect on sequence/affinity on assembly of peptide-heparin networks and effects on gelation time and mechanical properties [63].

This approach for delivery of heparin-binding growth factors has proven useful in a number of applications. Delivery of NGF and glial derived neurotrophic factor (GDNF) using fibrin scaffolds containing a heparin-binding delivery system enhanced peripheral nerve regeneration in a rat sciatic nerve injury model at both 6 and 12 weeks (see Figure 2) [64, 65]. In spinal cord injury models, delivery of NT-3 was found to promote neural fiber sprouting after acute and sub-acute (2 week delayed) treatment [66, 67].

This approach can also be expanded to allow not only delivery of exogenous growth factors, but for use as a method to sequester endogenous growth factors secreted *in vitro* or *in vivo*. Hudalla *et al*. have demonstrated that this approach can be used sequester endogenous growth factors from serum in culture [68] and to direct the differentiation of stem cells on self assembled monolayers [69]. Recently Seif-Naraghi *et al.* demonstrated that decelluarlized ECM can also serve as a vehicle for sequestration and delivery of endogenous heparin-binding growth factors [70].

HEPARIN MIMETIC POLYMERS FOR DRUG DELIVERY

Due to the heterogeneous structure of heparin, it would be beneficial to develop synthetic analogs that can bind to heparin-binding growth factors and provide more selective synthetic control of structure and thus allow better control of binding affinity. Maynard *et al.* used sulfated amino acids and combinatorial chemistry to identify artificial peptide sequences that function as heparin mimetics and can bind to heparin-binding growth factors [71]. They found a tetra-peptide sequence of sulfated amino acids that can bind to VEGF with micromolar affinity. This approach can be extended for nanopatterning applications using e-beam lithography to provide patterns of heparin-binding growth factors on a surface [72]. Nguyen *et al.* extended this work to demonstrate that co-polymers of styrene sulfonate and methyl methacrylate bearing PEG side chains could be used to stabilize bFGF and preserved its activity in the presence of environmental stressors, such as heat and acidic conditions [73]. This approach holds potential for developing sequences that are selective for an individual growth factors and that could potentially be resistant to enzymatic degradation by heparinases.

HEPARIN-MODIFIED MATERIALS TO DIRECT CELL DIFFERENTIATION

In addition to growth factor delivery, heparin-modified materials can also be used to direct cell differentiation of stem cell populations. For example, heparin-based delivery of growth factors (NT-3 and PDGFAA) was used to direct differentiation of embryonic stem cell (ESC)-derived neural progenitor cells *in vitro* and *in vivo* after spinal cord injury [74, 75]. Lam *et al.* used immobilization of bFGF and EGF via heparin on nanofibers to direct neuronal differentiation and axon growth of human ESC-derived neural stem cells *in vitro* [76]. Heparin-mimicking polymers made of polystyrene sulfonate (PSS) have been shown to promote myogenic differentiation of C2C12 muscle progenitor cells, see Figure 3 [77]. Delivery of bFGF from heparin/peptide amphiphiles was shown to promote survival, insulin secretion from and angiogenesis toward islets [78]. Heparin grafting onto biomaterials (scaffolds composed of polycaprolactone and polyhydroxybuterate) have been shown to

increase differentiation of induced pluripotent stem cells (iPSCs) into neuronal cells [79]. In other cases, the use of heparin-mimetic (PSS) surfaces can be used to increase the pluripotency of embryonic stem cell under the appropriate culture conditions [80]. Heparin-based hydrogels can also be used for the expansion of adipose-derived and bone marrow-derived stem cells [81]. In total, these studies suggest that heparin-containing or heparin-mimetic materials can play an important role in modulating the differentiation and pluripotency of stem cells.

HEPARIN-BASED MATERIALS

In addition to modification of materials with heparin covalently or based on affinity, heparin has been used more recently as a base polymer for the formation of hydrogels, and its ability to bind to basic peptides and cationic polymers has been exploited to promote self assembly of gels. Seal and Panitch demonstrated self assembly of PEG hydrogels by coupling heparin-binding peptides to each arm of a 4 arm PEG and mixing with heparin [82]. Zhang *et al.* built upon this approach and used a 4-arm PEG modified with either heparin-binding peptides from PF4 or low molecular weight heparin (as described above [51]) to form gels that assemble non-covalently and deliver bFGF [83]. Rajangam *et al.* used basic peptide amphiphiles and heparin to generate self assembling materials that provided controlled delivery of bFGF *in vitro* and angiogenesis in a rat corneal implant model [84]. This approach has been expanded to generate heparin/HA membranes with peptide amphiphiles to deliver VEGF, and these materials promote angiogenesis in a chick chorioallentoic membrane model [78, 85]. Some caution must be used with this approach, as the hydrogels formed using this method are generally weak mechanically, but may be useful for applications that require materials to gel after injection in the absence of any crosslinking agent or where materials are later crosslinked covalently by enzymes or via photo-polymerization.

This approach to drug delivery can be expanded to use heparin in combination with another polymer as the base material for the scaffold. Freudenberg used amine-terminated PEG star polymers to form hydrogels for growth factor delivery with heparin using EDC/NHS crosslinking. They also coupled Arginine-Glycine- Aspartic Acid (RGD) peptides to the heparin to enhance cell adhesion to the scaffolds. The addition of bFGF and RGD promoted improved cell survival and differentiation of mesencephalic neural stem cells from embryonic day 13.5 mice [86]. This work has continued on through a number of elegant studies that demonstrate the use of heparin-based materials to design Michael addition crosslinked and enzymatically degradable materials based on heparin-PEG star polymers that also facilitated delivery of growth factors [63, 87–92]. Recently, Freudenberg *et al.* have also demonstrated an elegant method for using modeling to predict and decouple the biomolecular and mechanical properties of heparin-based materials, see Figure 4 [93].

SUMMARY

Heparin modification of biomaterials have used for a broad spectrum of applications, including reducing material thrombogenecity, drug delivery, cell differentiation and promoting material self assembly. Incorporation of heparin into materials can be accomplished either via covalent conjugation or via non-covalent interactions with cationic polymers, including peptides and proteins. Methods for immobilization and conjugation have proven useful across many applications and have yielded a wide array of materials for biomaterial applications. In particular, non-covalent immobilization of heparin-binding growth factors, either exogenous or endogenous, in affinity-based delivery systems has proven high beneficial for many injury models and diseases.

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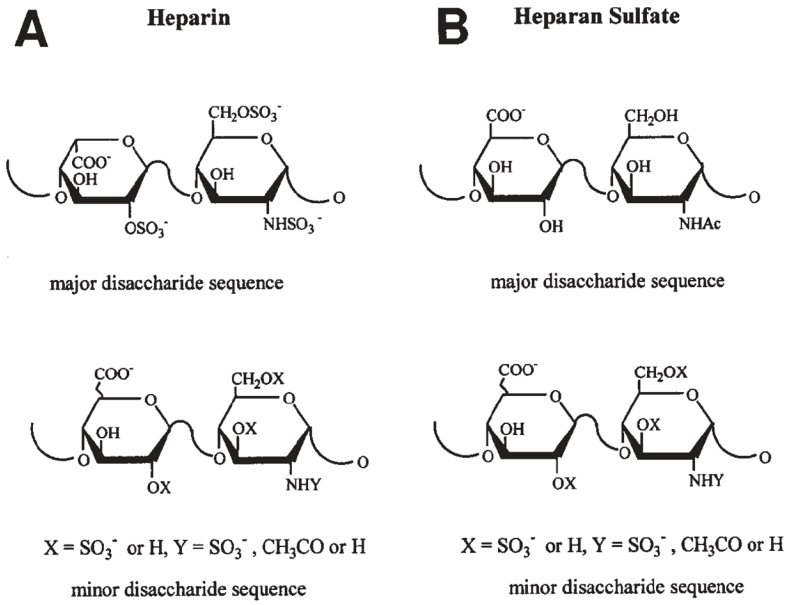

Figure 1.
(A) Structure of the major and minor disaccharide sequences of heparin. (B) Structure of the major and minor disaccharide sequences of heparan sulfate. Reprinted with permission from Ref. [94]

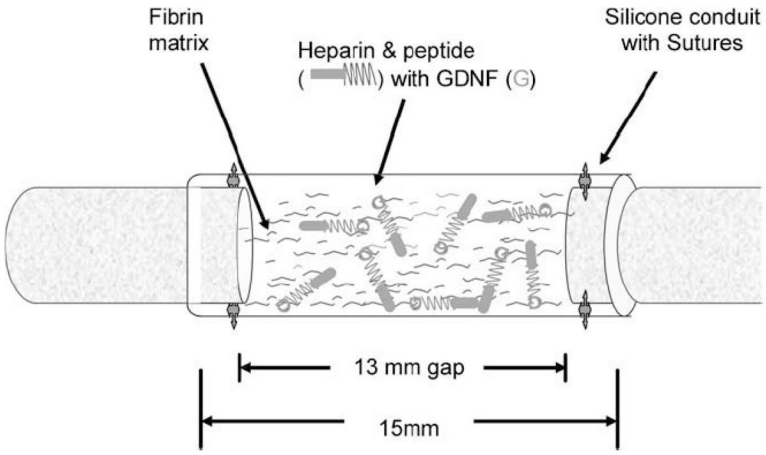

Figure 2.

Schematic representation of surgical implantation of nerve guidance conduit containing the affinity-based delivery system. A 13 mm nerve gap was repaired with a 15 mm silicone conduit containing fibrin matrices with or without delivery system and growth factor and sutured to the transected proximal and distal stumps, incorporating 1 mm of nerve on either end. The delivery system consisted of a bi-domain peptide crosslinked into the fibrin matrix at one domain while the other binds heparin by electrostatic interactions. The growth factor can then bind to the bound heparin, creating a matrix-bound, non-diffusible complex, which can be retained for cell-mediated degradation of the fibrin matrix. Reprinted with permission from Ref. [65].

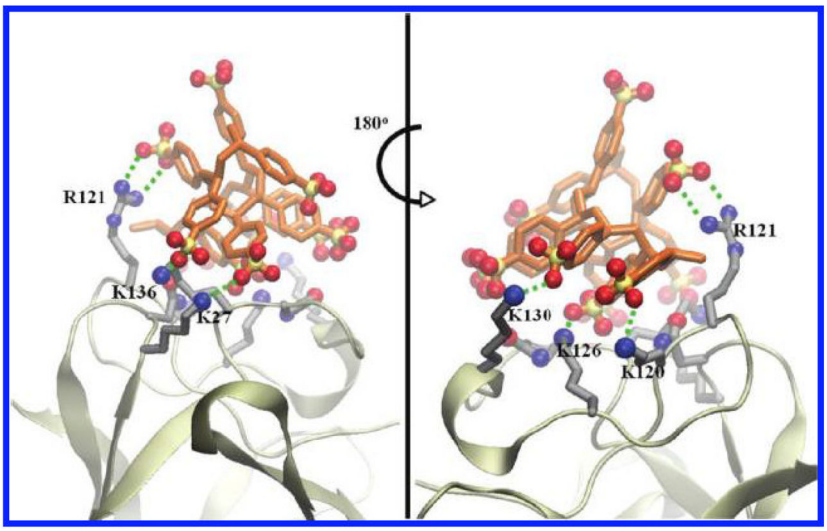

Figure 3.
Front and side views of the most favorable docked configuration of heparin mimetic polymer PSS on bFGF, illustrating salt bridges and hydrogen bonding interactions (green dashed lines) between the PSS and bFGF residues. Reprinted with permission from Ref. [77]. Copyright 2010 American Chemical Society.

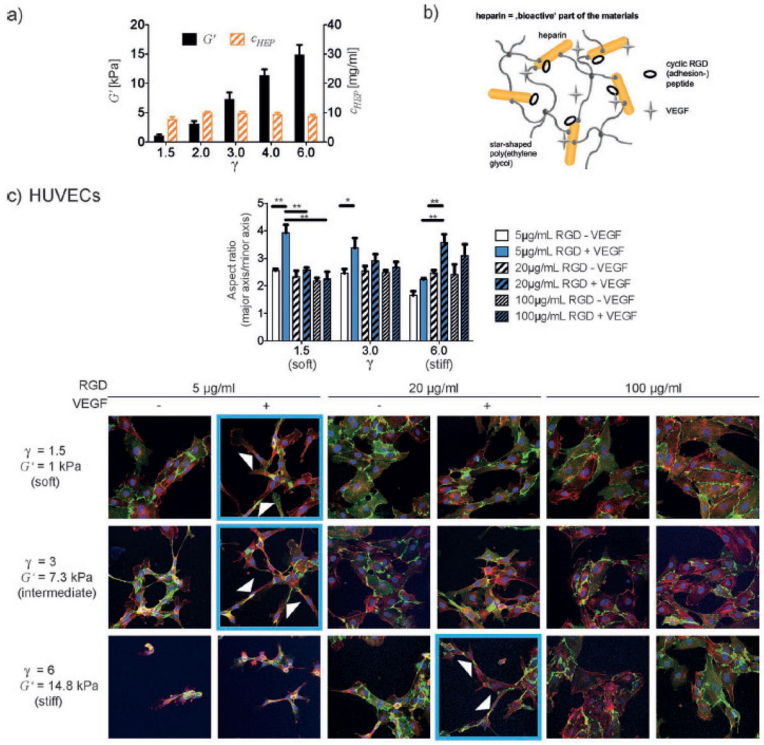

Figure 4.

a) Independent tuning of mechanical (indicated by the storage modulus) and biochemical (indicated by the constant heparin concentration) properties with varying γ. b) Heparin is the bioactive component of the hydrogel material mediating cell adhesion and provision of growth factors. c) HUVECs elongate to form a network of tube-like structures (arrows indicate cells with a high aspect ratio as a representative example) on starPEG-heparin hydrogels with independently varying VEGF and RGD incorporation and storage modulus. Images shown are confocal immunofluorescence images of CD31 (green, endothelial cell marker), actin (red), and DAPI (blue) of HUVECs plated for 20–24 h and are representative of results from 3 independent experiments. Mean values and standard error of the mean of three experiments in which > 20 cells each is shown (* $p < 0.05$ (significant), ** $p < 0.01$ (highly significant), p is the p -value of probability). Reprinted with permission from Ref. [93].