ABSTRACT

The present invention relates to a biologically active functionalized electrospun matrix to permit immobilization and long-term delivery of biologically active agents. In particular, the invention relates to a functionalized matrix composed of a matrix polymer, a compatibilizing polymer, and a biologically active molecule functionalized with a low molecular weight heparin. Examples of active molecules that may be used with the multicomponent polymer include, for example, a drug, a biopolymer, for example a growth factor, a protein, a peptide, a nucleotide, a polysaccharide, a biological macromolecule or the like. The invention is further directed to the formation of functionalized crosslinked matrices, such as hydrogels, that include at least one functionalized compatibilizing polymer capable of assembly.
FIG. 1

A

B

FIG. 2
FIG. 3

FIG. 4
FIG. 8
FIG. 9
FIG. 10
FIG. 11 (a–d)
FIG. 11 (e–h)
FIG. 13
FIG. 14
FIG. 15
FIG. 17
FIG. 18

FIG. 19
FIG. 20

G', G'' (Pa)

ω (Hz)

FIG. 21

G' 7.5:2.5
G' 9:0.5
G'' 7.5:2.5
G'' 9:0.5
G''

G' 25°C
G'' 25°C
G' 37°C
G'' 37°C
FIG. 22

FIG. 23
POLYMER MATRIX, USEFUL FOR THE CONTROLLED RELEASE OF A BIOLOGICAL APPLICATIONS, FOR EXAMPLE, IMPLANTABLE SYSTEMS FOR Molecule is rare, or difficult to purify. Furthermore, these insoluble, or unstable compounds. In addition, these investigation concerns the development of integrated systems macroscale (100-0.01 µm) or nanoscale (100-1 µm). One goal with these improvements there has been a move to miniaturize drug delivery devices from the macroscale (>1 mm) to the allow for the improvement of many existing therapeutics as well as enabling the use of entirely new therapies. Along with these improvements there has been a move to miniaturize drug delivery devices from the macroscale (>1 mm) to the microscale (100-0.01 µm) or nanoscale (100-1 nm). One goal of developing these devices is to provide for the in situ delivery of therapeutic compounds. Therefore, a main area of investigation concerns the development of integrated systems that combine the nanotechnology with therapeutic molecules, for example, drugs, or biopolymers. However, these new technologies must overcome several crucial and significant obstacles. For example, new approaches are required that allow for the delivery of insoluble, or unstable compounds. In addition, these approaches must address situations in which the therapeutic molecule is rare, or difficult to purify. Furthermore, these approaches must allow for localized delivery of potent compounds, and provide for improved compliance by reducing the chances of missing or erring in a dose. Lastly, these devices cannot be so small that they are unable to deliver an adequate dose.
can attach and migrate, be implanted with a combination of specific cell types as a cell delivery vehicle, and be utilized as a drug carrier to activate specific cellular function in the localized region.

The modification of biomaterials with bioactive molecules has been found useful in designing biomimetic scaffolds that can provide biological cues to elicit specific cellular responses and direct new tissue formation. The surface and bulk modification of materials with peptide sequences or with bioactive proteins or other biomolecules, for example, can allow for the modulation of cellular functions such as adhesion, proliferation and migration through alterations of peptide concentration or its spatial distribution. A variety of cell binding peptides have been introduced into three dimensional networks through physical, chemical, photochemical, and ioniccrosslinking. The molecules have been attached to a variety of polymeric substrates for varying applications, but a need still remains for methods for simple modification of materials with multiple bioactive molecules in a manner that can control their release.

Electrospinning is an attractive approach to polymer processing. The control of fiber diameter, porosity, and fiber surface morphology makes electrospun fibers useful in a range of applications including filtration, electronic, and biomedical applications. Electrospun fibers are ideal for use in biomedical applications such as tissue engineering and drug delivery due to the three-dimensional nanometer scale matrix that can be quickly produced using small quantities of starting material (<50 mg) on the laboratory scale. Larger matrices can also easily be produced via the scaling up of materials that prove to have useful materials and biological properties on the laboratory scale. Collagen, fibrinogen, chitosan, poly(lactic acid), poly(L-lactide-co-β-caprolactone), and poly(D,L-lactide-co-glycolide) are just a few of the polymers being investigated for use in electrospun drug delivery and tissue engineering constructs due to their biocompatibility. Electrospun fibers mimic the size scale of fibrous proteins found in the extracellular matrix (50-150 nm) and the three-dimensional nature of the matrix allows for cells to infiltrate the matrix and proliferate. A variety of additional polymers can also be electrospun into matrices for drug delivery, tissue engineering, and other applications. For example, see Z. M. Zhang, Y. Z. Zhang, M. K. Kotaki and S. Ramakrishna, Composites Sci. and Tech. 2003, 63, 2223-2253, (US Patent publication No. US20030137069).

Although there have been reports of the use of electrospun fibers as scaffolds for select drug delivery and biomaterials applications, the incorporation of bioactive molecules has not been widely exploited. In one example, tetracycline was included in a solution of poly(lactic acid) (PLLA) and poly(ethylene-co-vinyl acetate) and electrospun in a fiber membrane to study the release profile of the fiber-encapsulated drug. PLLA has also been studied as a matrix to incorporate the drugs rifampin (to treat tuberculosis) and paclitaxel (anticancer drug). Polymers loaded with both of these therapeutic compounds or via the reaction between amino-terminated poly($\varepsilon$-caprolactone) and amino groups on the amine reactive hydrogel networks (see, for example, N. Yamaguchi, K. L. Kiek, Polysaccharide-poly (ethylene glycol) star copolymers as scaffolds for the production of bioactive hydrogels, Biomacromolecules 6 (4) (2005) 1921-1930; N. Yamaguchi et al., Rheological characterization of polysaccharide-poly(ethylene glycol) star copolymer hydrogels, Biomacromolecules 6 (4) (2005) 1931-1940; B. L. Seal, A. Panitch, Physical polymer matrices based on affinity interactions between peptides and polysaccharides, Biomacromolecules 4 (6) (2003) 1572-1582).

Because hydrogels can mimic the high water content and mechanical properties of natural tissues, they are prime candidates as carriers of bioactive agents, in bioadhesive systems, or as biorecognizable materials. Given that poly (ethylene glycol), or PEG, is highly hydrophilic and generally nonadhesive to proteins or cells, it has found widespread use as a drug carrier, and many PEG hydrogels have been produced from aqueous solutions containing linear or branched PEG macromolecules via chemical crosslinking. In addition to hydrogels formed via radical crosslinking reactions, PEG hydrogels have been formed, for example, via Michael-type addition reactions upon mixing with thiol-bearing compounds or via the reaction between amino-terminated poly (ethylene glycol) and the herbal iridoid glycoside genipin. In some cases, cell adhesive peptide domains or biodegradable sequences have been introduced into PEG hydrogels to endow them more biological signaling functions, including the capacity for growth factor delivery. Accordingly, there have been a variety of covalently crosslinked hydrogel systems developed to deliver growth factors via mechanisms such as diffusion and chemical or enzymatic reaction.

Noncovalent interactions provide an alternative method for crosslinking, as introduced above, removing the need for toxic chemical crosslinking agents in gel preparation. In addi-
tion, noncovalent crosslinking strategies may offer advantages in maintaining protein integrity and bioactivity until delivery. Polymer hydrogels have been formed via specific recognition events such as reversible antibody-antigen interactions (see T. Miyata et al., *A reversibly antigen-responsive hydrogel*, Nature 399 (6738) (1999) 766-769) and coiled-coil interactions (see for example, C. Y. Xu et al., *Reversible hydrogels from self-assembling genetically engineered protein block copolymers*, Biomacromolecules 6 (3) (2005) 1739-1749; C. Wang et al., *Hybrid hydrogels assembled from synthetic polymers and coiled-coil protein domains*, Nature 397 (6718) (1999) 417-420; W. A. Petka, et al., *Reversible hydrogels from self-assembling artificial proteins*, Science 281 (5375) (1998) 389-392). There has been little attention given, however, to the interaction between peptides (particularly coiled-coils) and polysaccharides in hydrogel assembly. Further, the fabrication of biologically active fibers through such functionalization strategies is a fairly new and unexplored area in the field of electrospinning, and electrospun fibers have not been investigated for their ability to incorporate growth factor binding glycosaminoglycans, such as heparin, in a manner designed to control release.

SUMMARY OF THE INVENTION

The invention is directed to a functionalized polymer matrix comprising a matrix polymer, a compatibilizing polymer and a small functioning molecule. The invention is further directed to a method for formulating a functionalized matrix polymer comprising: functionalizing a compatibilizing polymer with a small functioning molecule and administering said functionalized compatibilizing polymer into a matrix polymer. The invention in also directed to a functionalized crosslinked matrix comprising at least one functionalized compatibilizing polymer capable of assembly. The invention is additionally directed to a crosslinked matrix that comprises the combination of a compatibilizing polymer-biomolecule conjugate and a growth factor. The invention is finally directed to a method for delivery of a drug to an animal comprising: formation of a functionalized matrix polymer comprising a matrix polymer, a compatibilizing polymer and a drug; and administering said polymer matrix to said animal.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a scanning electron micrograph of PEO/LMWH-dye electrospun fibers.

FIG. 2 shows Laser Scanning Confocal Microscopy (LSCM) images of electrospun PEO fibers (a) transmitted light and (b) fluorescence (5 µm scale bar).

FIG. 3 is an LSCM image of electrospun PEO/LMWH-dye fibers (5 µm scale bar).

FIG. 4 is a scanning electron micrograph of electrospun PEO/PLG-LMWH-dye fibers.

FIG. 5 is an LSCM image of PEG-LMWH-dye/PEO electrospun fibers (5 µm scale bar). Dark and colored regions are caused by folds in the surface of the macroscopic sample; not by composition variations.

FIG. 6 is showing scanning electron micrographs of (a) PLGA electrospun fibers, (b) PLGA/LMWH-dye fibers, and (c) PLGA/PEG-LMWH-dye fibers.

FIG. 7 shows LSCM images of electrospun PLGA fibers (a) transmitted light and (b) fluorescence (10 µm scale bars).

FIG. 8 shows LSCM images of electrospin (a) LMWH-dye/PLGA fibers and (b) PEG-LMWH-dye/PLGA fibers and (c) z-stack of PEG-LMWH/PLGA fibers (10 µm scale bars).

FIG. 9 shows bFGF binding on electrospun fibers. Each bar indicates standard deviation of the average of three measurements.

FIG. 10 shows scanning electron microscopy images of PEG-LMWH-dye/PLGA fibers and LMWH-dye/PLGA fibers after incubation in PBS for 6 h, 1 day, 3 days, and 14 days.

FIG. 11 shows retention of heparin within electrospun matrices as observed by multiphoton microscopy for the PEG-LMWH-dye/PLGA fibers and LMWH-dye/PLGA fibers after incubation in PBS for 6 h, 1 day, 3 days, and 14 days (10 µm scale bars).

FIG. 12 shows retention of PEG-LMWH-dye within depth of the electrospun matrix after 14 days of incubation in PBS as observed via multiphoton microscopy.

FIG. 13 depicts the release of bFGF from PLGA, LMWH-Dye, and PEG-LMWH-Dye electrospun fibers after 5 h and 24 h incubation in PBS.

FIG. 14 is a digital picture displaying the visual differences observed in the binding of bFGF to PLGA, LMWH-Dye, and PEG-LMWH-Dye electrospun fibers after 5 h and 24 h incubation in PBS.

FIG. 15 shows the results from the BrdU cell proliferation assay. Each bar indicates standard deviation of the average of three measurements.

FIG. 16 provides LSCM images of (a) LMWH/PLGA and (b) PEG-LMWLI/PLGA fibers (blue) incubated for 8 days with MG63 cells (green) (10 µm scale bars).

FIG. 17 shows (a) CD spectra of PEG-PF4ZIP at different concentrations in PBS at 25° C.; (b) Concentration dependence of the MRE for PEG-PF4ZIP at 222 nm. The solid line is the best fit.

FIG. 18 is a schematic of the assembly of hydrogels from solutions of the two polymer conjugates. The PF4ZIP is rendered as an oligomerized coiled-coil, as its concentration (200 µM) under these experimental conditions is two orders of magnitude greater than the measured Kd value.

FIG. 19 depicts storage moduli (closed symbols) and loss moduli (open symbols) of the PEG-LMWPA/PEG-PF4ZIP hydrogels for different molar ratios. The response of 2.5 wt % of the PEG-LMWPA (circles) is shown for comparison.

FIG. 20 shows plots of normalized storage moduli G' (ω=0.1 Hz) as a function of HBP(PF4ZIP, ATIII or HIP) to LMWH, HIP and ATIII data are adapted from reference 12. FIG. 21 is a comparison of storage moduli (closed symbols) and loss moduli (open symbols) of the PEG-LMWPA/PEG-PF4ZIP hydrogels (LMWPA/PF4ZIP=9:0.5) obtained at 25° C. and 37° C.

FIG. 22 shows shear recovery data for PEG-LMWPA/PEG-PF4ZIP hydrogels (LMWPA/PF4ZIP=9:0.5) at 25° C. and 37° C.

FIG. 23 depicts the bFGF release and hydrogel erosion profiles of the PEG-LMWPA/PEG-PF4ZIP hydrogel (LMWPA/PF4ZIP=8:1) and the PEG-LMWPA/PEG-HIP hydrogel (LMWPA:HIP 8:2). The errors are derived from the average of at least duplicate measurements.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates generally to a composition and method for the formation of functionalized polymer matrices comprising a matrix polymer, a compatibilizing polymer and a biomolecule or other small functionalizing molecule. The invention is a novel strategy for the incorpor-
The invention is currently demonstrated in a preferred embodiment comprising a low-molecular-weight-heparin (LMWH)-modified star copolymer incorporated into electrospun fibers. Attachment of active molecules to intermediate molecular weight polymers provides effective retention of the molecules in the material over time scales relevant for biological and other potential applications. The choice of intermediate molecular weight compatibilizing polymer will permit control over the retention and release rate of the molecule or other active species.

Advantages of using a polymer-conjugated biomolecule (i.e., polymer conjugate) for incorporation of bioactive molecules into materials as provided by the invention, in place of covalent attachment of the biomolecule to a high molecular weight polymer used directly in material formation, are several-fold. For instance, they include: i) the use of a polymer conjugate of the invention does not require a need for the development of distinct chemistry relevant for a particular molecule/polymer pair used in the material, as any polymer conjugate could be incorporated into any polymeric matrix; ii) the use of a polymer conjugate of the invention does not limit the number of molecules that could be incorporated into a given material for a given set of chemical transformations; iii) in biological applications, the incorporation of biomolecules via the current invention permits their controlled release in soluble form; iv) it is easier to control the release rate and inclusion of multiple molecules at varying ratios/amounts than when a bio (or other molecule) must be covalently attached directly to the polymers of the material; and v) the incorporation of biomolecules into materials via this invention allows not only control over release rates, but permits facile incorporation of multiple biological (or other active) species into materials, and offers the potential for each species to have a separate and distinct release rate.

The materials may find application in drug delivery or tissue engineering scaffolds to promote tissue repair and/or regeneration. They could be useful in other materials in related applications where controlled release of a multiple of active ingredients is desired, e.g., biosensors, detoxification, DNA delivery, etc.

An important, overriding difference of the invention from the prior art is that it allows for molecules (e.g., growth factors, proteins, peptides, and other non-biological molecules, etc.) to be incorporated into a polymer matrix via compatibilization of the molecules with the polymer matrix, and that it further allows for the controlled incorporation and release of a variety of different molecules from a polymeric matrix. For example, in the preferred embodiment (described in more detail in example 1), the incorporation of heparin, and more preferably LMWH, into an electrospun matrix via its attachment to a PEG polymer provides increased growth factor binding to the electrospun matrix. The increased growth factor binding is due to the conjugation of LMWH to the PEG molecule. The PEG-LMWH molecule takes longer to diffuse out of the matrix, therefore allowing for more growth factor binding to occur within a given time period as shown by comparing the amount of growth factor binding to the PEG-LMWH matrix versus a matrix consisting of LMWH alone (See Example 1).

In the case of electrospun matrices, while there are limitations to what polymers can be electrospun, there are many possible uses. Solubility of fibers must be controlled, but such control is also possible with this invention as well as matching of mechanical properties for specific applications that are accomplished using appropriate choice of polymer, additives, and/or processing protocols.

### Matrix Polymer

Matrix polymers, effective in this invention include but are not limited to: Nylon 6,6; Nylon 6,10; (other nylons, also), polyurethanes, polyacrylonitrile, polyvinyl alcohol, polyacrylic acid, polyethylene-co-vinyl acetate, polycarbonate, poly(2-hydroxyethyl methacrylate) (polyHEMA), poly(vinylidene fluoride), poly(vinylidene chloride), poly(ethylene glycol)/PEG, polyvinyl pyrrolidone, polyethylene, polycaprolactone, poly(3-hydroxybutyrate), poly(ortho esters), polyhydroxides, poly(ether-ester) azopolymers, poly(dimethyl siloxane), poly(phosphazenes), other copolymers of the above homopolymers (e.g., poly(methacrylic acid-co-ethylene glycol), and others.

Matrix polymers may also comprise natural polymers, such as collagen, silk, silk-like protein polymers, elastin, elastin-like protein polymers, poly(aminic acids), cellulose acetate, hyaluronic acid, chitosan, fibronectin, and others.

Composite materials comprising any of the above polymers and an appropriate inorganic matrix (clays, silica, microparticles, etc.) can also be employed as a matrix polymer for applications requiring high mechanical strength, such as hard tissue engineering applications.

For drug delivery and biological applications, the following are particularly preferred: Poly(ethylene), poly(propylene), poly(vinyl chloride), poly(vinyl alcohol), poly(ethylene-vinyl acetate), poly(enol-ketone), poly(acrylic acid), poly(acrylamides), poly(N-alkyl acrylamides), poly(alkyl acrylates), PEG, PEO, poly(tetrafluoroethylene), poly(glycolic acid), poly(lactic acid), poly(lactic-co-glycolic acid), poly(caprolactone), poly(3-hydroxybutyrate), poly(ortho esters), poly(aryldiones), poly(ether-ester) azopolymers, poly(dimethyl siloxane), poly(phosphazenes), among others. In addition, collagen, silk, silk-like protein polymers, elastin, elastin-like protein polymers, poly(aminic acids), cellulose acetate, hyaluronic acid, chitosan, fibronectin, and others, may also be used.

For applications in which a molecule is to be released in a non-biological environment (e.g., water purification, agricultural treatments, biosensors, smart fabrics, etc.), any of the above polymers could be employed, specific polymers would be chosen on the basis of the specific application.

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For applications in which a molecule is to be released in a non-biological environment (e.g., water purification, agricultural treatments, biosensors, smart fabrics, etc.), any of the above polymers could be employed, specific polymers would be chosen on the basis of the specific application.
Compatibilizing Polymer

Any moderate molecular weight polymer, of the chemical compositions of the above matrix polymers, that can be modified via reasonable chemical means with a biomolecule or other small functional molecule, moiety, or particle is considered a compatibilizing polymer according to this invention. Moderate molecular weight preferably comprises, but is not limited to, a molecular weight of approximately 100 to 5000 the molecular weight of the matrix polymer it is bound to, in such a range that the compatibilizing polymer permits homogeneous incorporation of the biomolecule into the matrix polymer and sufficiently slows the diffusion-based release of the biomolecule over timescales relevant for a particular application. Some examples of moderate molecular weight polymers that would function effectively as a compatibilizing polymer in this invention include synthetic degradable polymers such as poly(glycolic acid), poly(lactic acid), poly(ethylene glycol) (PEG), poly(lactide-co-glycolide), and poly(caprolactone) and others.

Additional polymers include polymers that could function as the compatibilizing polymer such as: poly(acrylic acids), poly(acrylamides), poly(N-alkyl acrylamides), poly(alkyl acrylates), poly(3-hydroxybutyrate), poly(ortho esters), poly(ortho-hydrides), poly(amido amines), poly(methacrylates), poly(dimethyl siloxane), among others. In addition, collagen, silk, silk-like protein polymers, elastin, elastin-like protein polymers, poly(amino acids), cellulose acetate, hyaluronic acid, chitosan, fibronectin, and others, may also be used. As the compatibilizing polymer can be functionalized at chemical groups located along the length of the polymer chain or at the termini of the polymer, many polymers can be employed. Potential additional polymers include: Nylon 6,6; Nylon 6,10; (other nylons, also), polyurethanes, polyacrylonitrile, polyvinyl alcohol, polylethylene-co-vinyl acetate, polycarbonate, poly(methacrylates), poly(alkyl methacrylic acids), polycarboxylic acids, poly(alkyl acrylate), poly(N,N'-diethylaminomethyl methacrylate), poly(esteramide) terephthalate, polyestrene, polyvinyl chloride, polyvinyl phenol, polyacrylamide, poly(alkyl acrylamide), poly(2-hydroxyethyl methacrylate) (polyHEMA), poly(vinylidene fluoride), poly(vinylidene chloride), poly(vinyl pyrrolidone, poly(3-hydroxybutyrate), poly(anhydrides), poly(ether-ester) azopolymers, poly(dimethyl siloxane), poly(phosphazenes), other copolymers of the above homopolymers (e.g. poly(methacrylic acid-co-ethylene glycol), and others.

A preferred compatibilizing polymer for use in this invention is poly(ethylene glycol) or PEG. PEG is a linear or branched neutral polymer with the chemical formula HO—(CH₂CH₂)n—O. PEG is highly soluble in water and many organic solvents (e.g., methylene chloride, ethanol, toluene, acetone, and chloroform), and is readily available in various sizes and functionalized architectures (e.g., amino-, carboxyl-, and sulfhydryl-terminated). Low-molecular-weight (<100 daltons) PEGs are viscous and colorless liquids, whereas high-molecular-weight PEGs are waxy solids whose melting points are proportional to their molecular weights with an upper limit near 300 °C. PEG has been found to be nontoxic and is approved by the FDA for use in drugs (parentals, topical, suppositories, nasal sprays), foods, and cosmetics. Molecular weights of PEG used in biomedical applications usually range between a few hundred to 20,000 Da. The molecular weight distribution of these PEG polymers is generally low, but the commonly used mono-methyl ethers of PEG exhibit broader molecular weight distributions. In solution, PEG is also a highly hydrated polymer, where each monomer (ethylene oxide unit) can bind three molecules of water. PEG polymer exhibits a large degree of segmental flexibility in aqueous solution and it is thought that PEG has the ability to influence the structure of several molecular layers of more loosely associated hydrated water molecules.

PEG is valued for its ability to exclude other polymers (natural and synthetic) from its presence in aqueous environment. This exclusion of other polymers is the primary driving force behind PEG's ability to reject proteins, from two-phase systems with other synthetic polymers, and makes this polymer both nonimmunogenic and nonantigenic. Because of its many benefits, PEG is ideally suited for protein modification. Indeed, PEG is currently considered one of the most successful techniques to prolong the residence time of protein drugs in the bloodstream. There are dozens of examples of PEG-protein conjugates described in the literature. For example, PEG has been considered a tool to enhance drug delivery to neoplastic tissues such as, for instance, pegylated monoclonal antibodies or MoAb fragments have been investigated as immunotargeting agents. (see, for example Sato H., Enzymatic procedure for site-specific pegylation of proteins, ADVANCED DRUG DELIVERY REVIEWS 54 (4): 487-504 Jun. 17 2002; Deliars A., et al., Site-specific PEGylation of proteins containing unnatural amino acids, BIOORGANIC & MEDICINAL CHEMISTRY LETTERS 14 (23): 5743-5745 Dec. 6 2004; Hermanson, Greg T., Bioconjugate Techniques, 1st edition, Academic Press, New York, 1996) (All of which are herein incorporated by reference in their entirety).

PEG conjugation technology has been used, for example, to improve the therapeutic efficacy of recombinant human proteins because it alleviates many problems associated with therapeutic protein formulations. For example, the attachment of PEG to a protein affects its molecular size, charge, and receptor-binding capabilities, which can serve to decrease the conjugate's overall rate of clearance from the body. Also, by sterically shielding the protein domains susceptible to proteolytic attack, PEG decreases the protein degradation that causes protein biological inactivity. Finally, by sterically masking a therapeutic protein’s immunogenicity/antigenic determinants. PEG conjugation commonly results in conjugates that are nonimmunogenic and nonantigenic. The result of changes in the parental protein’s characteristics by PEG almost invariably increases the plasma half-life and resistance to proteolytic degradation, and decreases immunogenicity and antigenicity of the resultant PEG-protein conjugate. Although PEG is generally considered to be non-immunogenic, its parenteral administration has been found to stimulate the production of antibodies.

Small Functioning Molecule

Small functioning molecules according to this invention include, but are not limited to, biomolecules and functional molecules such as peptides, sulfated peptides/polypeptides, peptides/polypeptides with other functionalization/modifications, peptides/polypeptides containing non-natural amino acids, polypeptides (natural AA, sulfated, non-natural AA, other modified side chains, etc.), protein fragments, proteins (e.g., growth factors, growth hormones, cytokines, insulin, etc.), enzymes, saccharides, oligosaccharides, polysaccharides, small molecule drugs (e.g., dioxorubicin, aspirin, adriamycin, tetraacycline (antibiotic), chloroquine (antimalarial), tamoxifen, and other small molecule drugs that can be chemically conjugated to a polymer), dyes, organics, metals, and nanoparticles and nanomolecules.

One particularly interesting biomolecule is heparin. Heparin is a highly sulfated glycosaminoglycan that binds growth
factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), heparin-binding epidermal growth factor (HBEGF), transforming growth factor-β (TGF-β), and others. Those having skill in the art are clearly aware that growth factors are important for controlling cellular activities such as cell growth and proliferation. The incorporation of heparin in the electrospun matrix is of functional importance owing to heparin’s role as a binding site for many growth factors; sulfated peptides may have similar activity and would therefore also be useful for similar reasons. For example, the binding of growth factors to glycosaminoglycans in the ECM is essential for storage, release, and preservation of the bioactivity of the growth factor. Heparin primarily serves to immobilize growth factors until release but is also known to control their mitogenic activity, and in several cases, serves as a cofactor to promote binding of the growth factors to their receptors. Heparin is also known to regulate the growth of fibroblast and endothelial cells when combined with heparin-binding growth factors. For example, heparin has been reported to have been incorporated into a chitosan hydrogel where it was found to induce neovascularization in vivo and enhanced the activity of acidic fibroblast growth factor (aFGF) and basic fibroblast growth factor (bFGF) and stabilized the growth factors in vitro. A variety of other reports also confirm the benefits of including heparin in a bulk hydrogel matrices, such as those comprising fibrin, PEG, collagen, hyaluronic acid, and synthetic polymers, and the extension to a fibrous matrix may provide additional structural advantages for producing materials that mimic the ECM. Incorporation of biomolecules such as heparin, into a polymer matrix allows for the exploitation of heparin’s affinity for growth factors as a strategy to deliver them from a fibrous ECM-mimetic matrix. Those having skill in the art understand that heparin binds various proteins with disparate function. It is contemplated that any such protein would be candidates for use in this invention.

Method of Formulating Functionalized Polymers

Some methods for processing polymers into a given form that can be applied in the invention for producing polymeric matrices capable of controlled release of the compatibilizing polymer/biomolecule conjugates include, for example, crosslinking of a polymer to form a hydrogel network can be employed; any method for crosslinking polymers can be applied for formation of the crosslinked network, using standard initiators or at least trifunctional monomers; standard free radical initiators include thermal initiators, redox initiators, photoinitiators; the inclusion of a bifunctional vinyl molecule with appropriate vinyl monomers would produce crosslinked networks of the vinyl matrix polymers. Additionally, noncovalent methods of producing a crosslinked network could be employed. Such interactions include but are not limited to ionic crosslinking (e.g. alginic crosslinking via interaction with divalent cations, layer-by-layer deposition of polyelectrolytes, etc.), protein–protein interactions, peptide-saccharide interactions, saccharide-saccharide interactions, self-assembly of peptidic molecules, and antibody-antigen interactions. The pore size of the crosslinked network should be smaller than the size of the compatibilizing polymer/biomolecule conjugate in order to permit release of the biomolecule to be delayed via its conjugation to the compatibilizing polymer.

Any method of forming different three-dimensional particles and structures could also be applied in the current invention. For example, emulsion (or inverse emulsion) polymerizations, crosslinking (in colloidal suspensions, micellar or liposomal solutions, or emulsions), or spray crosslinking can be used to form particulate and nanoparticulate polymers. Crosslinking of monomer solutions in a mold or microfluidic channel permits the construction of a matrix of essentially any shape/size. Films cast of the monomer solutions (e.g., via spincoating, doctor blade application, diecasting, etc.) can also be produced via crosslinking by any of the above mechanisms or via simple casting/drying of a non-water soluble polymer.

Electrostatic fiber spinning, or “electrospinning,” is a preferred process for this invention. Electrospinning is a process by which superfine fibers, ranging from approximately 10 nanometers to about 10 microns in diameter, are formed. The resulting electrospun fiber web has a high specific surface area that is beneficial in a wide variety of technologies due to the small fiber diameter and its porous structure. A high voltage source is used during the electrospinning process to generate an electrically charged jet of polymer solution or melt expelled from a device such as a pipette. This process is then followed by a drawing of the resulting filaments as they solidify or coagulate. Electrospinning provides a simple approach for production of solid, as well as hollow, one-dimensional nanofibers from a variety of starting materials. To date, dozens of synthetic or natural polymers, melts, and polymer blends have been successfully spun using the electrospinning process. (see, for example, Shin et al., Biomaterials 2003, 24, 4353–4364; Suh et al., Polymer 2003, 44, 5721–5727 (Frank Ko); Li et al., J. Biomed. Mater. Res. 2002, 60, 613–621 (Frank Ko); Jin et al., Biomacromolecules 2002, 3, 1233–1239 (David Kaplan); Jin et al., Biomaterials, 2004, 25, 1039–1047 (David Kaplan); Fanuc-Carson, M. C.; Wagner, R. C.; Krick, K. L., Extracellular Matrix: Structure, Function, and Applications to Tissue Engineering, CRC Biomedical Engineering Handbook, CRC Press, New York, 2004; Huang et al., Composites Science and Technology 2003, 63, 2223–2253) (All said references are herein incorporated by reference in their entirety).

A broad range of electrospun materials have been published and include synthetic polymers with electrical properties ranging from non-conducting polyurethane to conducting polyaniline, in addition to natural polymers such as collagen and silk worm silk. There is also much recent work on nanofibers electrospun from biocompatible or biodegradable polymers for bioengineering applications. The fibers may be made from a polymer solution via an electro spinning process as described in Reneker, U.S. Pat. No. 4,323,525, U.S. Pat. No. 4,689,525, US 20050195611, US 20040018226, and US 20010045547, which are incorporated herein by reference in their entirety. The specific types of polymers that may be produced in fiber form via the electrospinning process include those listed in Huang, U.S. 20030195611, U.S. 20040037813, U.S. 20030138014, U.S. 20040013878, U.S. 20030121792, U.S. 200301215624, US 20030195611, US 20030168756, US 20030106294, US 20020175449, US 20020100725 and US 200200084179 all of which are herein incorporated by reference in their entirety.

When constructing electrospun fibers for biomedical applications, certain material considerations must be acknowledged. Some minimal properties of a drug delivery system are that the material must be non-immunogenic, degradable with non-toxic products, able to be excreted from the body, and deliver therapeutic molecules in a controlled and predictable manner. Manufacturing time and complexity, along with handling, must also be considered. In compliance with these requirements, matrix polymers such as PEO and PLGA serve as excellent candidates for fabrication of the matrix via electrospinning; indeed, the successful use of these polymers in a variety of tissue engineering and drug delivery applications.
has been documented. Those having skill in the art are familiar with the processes used in tissue engineering and drug delivery as required for incorporation in this invention.

In addition to electrospinning, other traditional methods of making polymeric fibers can be applied, when possible, to the formation of active fibers of larger diameter. Given the choice of biomolecule or functional molecule or particle with a particular matrix polymer would dictate the use of solution spinning, melt spinning, or other spinning protocols.

Regarding the attachment of the biomolecule (or other small functioning molecules) to the compatibilizing polymer, the chemical reactions to be used are unlimited and are chosen based on the desired compatibilizing polymer/biomolecule combination. Initially, the matrix polymer composition may be altered for any given desired application and the combinations of matrix polymer/compatibilizing polymer/biomolecule for any application are limited only by the choice of a given application, not by the scope of the invention.

In a preferred embodiment, poly(ethylene glycol) (PEG) functionalized with low molecular weight heparin (PEG-LMWH), is fabricated into fibers for use in drug delivery, tissue engineering, or wound repair applications. In particular, a four-arm star PEG with covalently attached LMWH was found to be well suited for incorporation into electrospun fibers with matrix polymers such as PEO and PLGA. Further, the use of a PEG-LMWH conjugate proved to have a better effect on growth factor binding than when compared to free LMWH (see Example 1).

Interestingly, it was observed that the incorporation of heparin into the electrospun PEO and PLGA fibers did not affect the surface morphology or fiber diameters. The fibers produced had diameters ranging from approximately 100 nm to 400 nm. Our data indicates that PEG-LMWH conjugates can be incorporated into an electrospun matrix at concentrations ranging from 3.5 µg to 85 µg per milligram of electrospun fibers, although with greater heparin concentrations in the spinning solution, it should be feasible to produce higher levels of heparin loading in the fibers. Incorporation of PEG-LMWH into the matrix permits retention of the heparin for at least 14 days. Improvements in the binding of basic fibroblast growth factor (bFGF) to the electrospun fibers were also observed for fibers functionalized with PEG-LMWH over those functionalized with LMWH alone. The combination of these results suggests the utility for producing electrospun fibers that are appropriately functionalized for use in biomaterials applications.

Although LMWH was indicated to be incorporated into fibers with simple mixing in our investigation, the conjugation of LMWH to the star PEG slightly improved the amount of heparin incorporated into the fiber matrix and permitted a significantly improved amount of growth factor binding to these matrices, which was suggested to result from an improved retention of heparin in the fibers. If the use of such PEG-LMWH conjugates also slows the release of heparin over extended periods of time, it will allow for a slower, more sustained release of growth factor, which is often required to achieve desired biological outcomes.

The conjugation of heparin, and more preferably, LMWH, to a relatively short (relative to the matrix polymer molecular weight) polymeric chain therefore permits facile functionalization of fibers and also allows for retention and significantly slowed release of LMWH from the matrix over time. Given the importance of co-presentation of the LMWH with growth factors at the growth factor receptor these systems also offer the potential advantage of improving growth factor activity over systems in which heparin is covalently attached directly to the matrix and can therefore not be co-released with growth factor. The rate of release of heparin (and therefore growth factors) may also be modified via modifications in the polymer conjugate's molecular weight over a wide range, and coupled with the greater entanglement likely for flexible polymer chains, this may offer advantages over simple incorporation of a higher molecular weight heparin in fibers. In addition, the controlled incorporation and delivery of other and potentially smaller bioactive molecules, such as peptides, would also be widely possible via the incorporation of other similar PEG bioconjugates into these fibers. The ability to simply mix various PEG-conjugates in the electrospinning solution also offers advantages for creating multifunctional fibers.

The PEG-LMWH molecules used in these electrospinning studies have previously been demonstrated to be capable of sequestering growth factors in hydrogels and delivering them in bioactive form to induce proliferation of human umbilical vein endothelial cells. The amount of LMWH in these electrospun fiber matrices (3-86 µg) is far higher than hydrogels produced with these polymers; the hydrogels can effectively release bFGF for time periods of at least 16 days. These results therefore show significant promise for the potential of delivering similarly active growth factors from electrospun matrices. The additional advantage of the ease of mixing combinations of PEG-conjugates in the fibers is also promising for the facile production of multifunctional, non-immunogenic matrices for biomaterial applications.

It was found that LMWH functionalized PEG molecules can be easily incorporated into electrospun fibers. These fibers have nanometer scale diameters when electrospun using either PEO or PLGA as carrier polymers. The amount of LMWH per mg of fibers can range from 3.5-9.5 µg depending on the sample examined. Although both LMWH and PEG-LMWH can be processed into fibers, the incorporation of PEG-LMWH results in functional advantages such as improved growth factor binding, resulting from improvements in retention of the PEG-LMWH in the fibers over the LMWH alone. The PEG-LMWH can be retained in the fibers for at least 14 days, in contrast to LMWH, which is almost completely released from the fibers after 24 hours. A slower release of LMWH, and any coupled growth factors, allows for drug delivery over a period of time that more closely matches the time scales needed for tissue repair or regeneration should the fibers be used in biomedical applications. The use of electrospinning as the fabrication method for this biologically functionalized matrix is preferred due to its ability to allow for the incorporation of LMWH, ease of fabrication, fiber spinning at a variety of temperatures, and use of small amounts of material. The ability to scale electrospinning from the laboratory to the industrial scale offers opportunities to produce matrices for biomaterials applications. The electrospun matrix also meets many requirements in its two-dimensional and 3-D nature to make the fibers suitable for drug targeting and tissue engineering applications. Such simple strategies for creating electrospun matrices that are both structurally relevant and biologically active could find increased utility in a variety of biomedical applications.

Functionalized Crosslinked Matrices

In an alternative embodiment, the invention is directed to the use of a functionalized compatibilizing polymer in the production of materials in which assembly, mechanical response, and biological properties are controlled by protein-polysaccharide interactions. Such materials are designed to mimic the biological environment and find use in the delivery of growth factors.
Specifically, functionalized crosslinked matrices, such as hydrogels, capable of assembly by, for example, interactions between heparin and peptides, permit the formation of matrices that allow for effective protein delivery. For example, an elastic or viscoelastic hydrogel formed by the combination of a compatibilizing polymer-LMWH conjugate and a compatibilizing polymer-peptide conjugate is a preferred interaction contemplated herein. While the use of any compatibilizing polymer is contemplated, a preferred compatibilizing polymer for use in this embodiment would include PEG. Also, while preferred, it is not necessary that the compatibilizing polymer be the same for both the LMWH conjugate and the peptide conjugate.

In this particular embodiment, a hydrogel provides the benefits of controlling drug delivery rates by, for example, changing the heparin-binding peptide or manipulating the hydrogel’s physical properties and erosion profiles.

In a specifically preferred embodiment (more fully described in Example 4) a heparin-binding, coiled coil peptide, PF4ZIP, was employed to mediate the assembly of heparinized polymers. Heparin binding peptides (HBP) from antithrombin III and the heparin interacting protein have also been employed; any heparin-binding peptide with desired affinities can be used in the production of materials of this kind. Importantly, viscoelastic hydrogels were formed upon the association of PF4ZIP-functionalized star poly(ethylene glycol) (PEG-PF4ZIP) with low-molecular-weight heparin-functionalized star PEG (PEG-LMW). The viscoelastic properties of the hydrogels can be altered via variations in the ratio of LMWH to PF4ZIP (or any HBP). Comparison of growth factor (bFGF) release profiles with the hydrogel erosion profiles indicates that bFGF delivery from this class of hydrogels is mainly an erosion-controlled process and the rates of bFGF release can be modulated via choice of HBP or via variations in the mechanical properties of the hydrogels. Manipulation of hydrogel physical properties and erosion profiles provides novel materials for controlled growth factor delivery and other biomedical applications.

Growth Factor-Crosslinked Matrices

In even another alternative embodiment, the invention is directed to the use of a functionalized compatibilizing polymer to form a crosslinked matrix that comprises the combination of a compatibilizing polymer-biomolecule conjugate and a growth factor, wherein a biomolecule such as LMWH functions effectively. In such an embodiment, it is intended that the growth factor function as a crosslink that eventually is removed and drug is released. Removal of the growth factor may result from passive release and/or targeted release via targeted ligand exchange of the growth factor with specific receptors on cell surfaces. The growth factor crosslink may result from passive release and/or targeted release via similar mechanisms should therefore also be possible to permit release of multiple, bioactive growth factors.

Although the invention is illustrated and described herein with reference to specific embodiments, the invention is not intended to be limited to the details shown. Rather, various modifications may be made in the details within the scope and range of equivalents of the claims and without departing from the invention.

EXAMPLES

Electrospinning Functionalized Fibers

Materials and Methods

Hydroxy terminated and thiol terminated four-arm star poly(ethylene glycol) (Mw=10,300; Mn=11,300 g/mol and Mw=10,000; Mn=10,800 g/mol, respectively) were purchased from Polymer Source (Dorval, QC, Canada). LMWH sodium salt (porcine intestinal mucosa, avg. molecular weight 3000 g/mol) was obtained from Sigma (St. Louis, Mo.). Alexa Fluor® 350 carboxylic acid, succinimidyl ester was obtained from Molecular Probes (Eugene, Oreg.). Solvents and reagents were distilled or purified according to standard procedures.

Synthesis of PEG-LMW Conjugate

N-Decacylation of LMWH

LMWH (Mw=3000 g/mol) was used in these investigations. In some cases, the LMWH was first separated into a high affinity fraction via anionic exchange chromatography on diethyl aminomethyl (DEAE) resin. Such fractionalization is not an absolute requirement, but provides a LMWH fraction with higher heparin binding affinity. LMWH was eluted from the column via gradient elution in Tris buffer (pH 7.5, 20 mM) with sodium chloride concentrations ranging from 0 to 2M. The late eluting fraction of LMWH was collected, dialyzed against several changes of deionized water (Spectrapor, MWCO 1000 Da), and lyophilized prior to use. The fractionated LMWH (250 mg), hydrazine (5.3 g), hydrazine sulfate (53 mg), and water (2.3 mL) were added to a heavy duty reaction vessel, and the reaction vessel was tightly capped and the mixture was stirred at 95°C for 16 h. After the solvents were removed in vacuo, the product was dialyzed using a Spectrapor dialysis membrane (MWCO 1000 Da) first against 1 M NaCl and finally against water (for each dialysis bath was changed four times after 2, 8, 16, and 24 h) and lyophilized to afford a slightly yellowish solid, 153 mg (61% yield).

Synthesis of Maleimide and Alexa Fluor 350® Functionalized LMWH

To a 10 mL vial were added N-deacetylated heparin (10 mg), dry Dowex 50WX4-200 ion-exchange resin (strongly acidic cation), and anhydrous dimethylformamide (DMF, 4 mL) and the reaction mixture was stirred at room temperature until complete dissolution of heparin was observed. The resin was filtered and to the filtrate were added a few drops of
anhydrous triethylamine and a solution of 4-(N-maleimidomethyl)cyclohexanecarboxylic acid N-hydroxysuccinimide ester (0.75 equiv.) (Sigma, St. Louis, Mo.) in anhydrous DMF (0.3 mL) and the reaction mixture was stirred at 60°C for 20 h. To this mixture was added a solution of Alexa Fluor 350 carboxylic acid, succinimidyl ester (1 equiv.) in anhydrous DMF and the reaction mixture was further stirred at 60°C for 20 h. The solvent was removed in vacuo to yield a slightly yellowish solid, which was redissolved in PBS (pH 6.6, 0.10 M, 0.15 M NaCl). After the insoluble particles were filtered, the filtrate was purified via fast protein liquid chromatography (FPLC) equipped with a Superdex 200 gel filtration column (Amersham, Piscataway, N.J.) using 0.2 M NaCl at 0.5 mL/min as eluting buffer. The heparin containing fractions were combined and dialyzed using a SpectraPor membrane (MWCO 1000 Da) first with 1 M NaCl and finally with water (each dialysis bath was changed four times after 2, 8, 16, and 24 h) and were then lyophilized to give a slightly yellowish solid, 4.2 mg. 1H NMR (400 MHz, D2O, 29°C): δ: 0.86-1.95 (10H, cyclohexyl, m), 3.38-5.43 (72H, heparin, m), and 6.84 ppm (2H, —COOH—CH₂CO, m).

Synthesis of PEG-LMWH

To a 100 mL round bottom flask equipped with a N₂ inlet were added maleimide and dye-functionalized reagent. The mixture was degassed by bubbling N₂ for 15 min. To this solution was slowly added a degassed solution of PBS containing thiol terminated four-arm star PEG (65 mg in 10 mL) and the reaction mixture was stirred at room temperature for 2 h. The reaction of the thiol was 96% complete as confirmed via analysis with Ellman’s reagent. The reaction mixture was quenched by adding a solution of N-ethylmaleimide (12 mg) in DMF (0.4 mL). The crude product was purified via FPLC with a Superdex 200 gel filtration column (Amersham, Piscataway, N.J.) at 0.5 mL/min using 0.2 M NaCl as eluting buffer. The product isolated by FPLC was purified via dialysis against water (water bath was changed four times after 2, 8, 16, and 24 h) and lyophilized to give a slightly yellowish solid, 150 mg. 1H NMR (400 MHz, D2O, 29°C): δ: 0.86-1.95 (40H, cyclohexyl, m), 2.50-2.88 (12H, —COCH₂CH₂CO, m), 3.38-5.43 (288H, heparin, m), and 3.70 ppm (908H, PEG backbone, —CH₂CH₂O, m). Integration of the 1H NMR spectrum suggests 75% functionalization of PEG with LMWH.

Electrospinning and Characterization

Fiber Spinning

The electrospinning apparatus consisted of a syringe pump (Orion Sage, Fisher, Fair Lawn, N.J.), high voltage supply (Glassman Series EH, High Bridge, N.J.), 1 mL syringe (Piper & Sons, New Hyde Park, N.Y.) and a needle (Hamilton, Reno, Nev.) with an inner diameter of 0.51 mm. A voltage of −12 kV was applied to the tip of the needle. A grounded metal sheet was used as the target and was positioned 15 cm from the tip of the needle for sample collection. The concentration of the polymeric solutions varied depending on the carrier fluid employed. PEO (BDH Chemicals, Poole, UK, Mn = 300,000 g/mol) was used at a 10 wt % concentration in water and a flow rate of 0.07 mL/min was employed. PLGA (Sigma Aldrich, St. Louis, Mo., Mn = 90,000-126,000 g/mol) solutions were made at a 45 wt % concentration in DMF (Fisher, Fair Lawn, N.J.) and required a flow rate of 0.26 mL/hr.

Characterization

The electrospun fibers were characterized via field emission scanning electron microscopy (FESEM) (Hitachi S-4700) coupled with the energy dispersive X-ray (EDX) (Oxford Inca X-Ray Microanalysis) for elemental analysis of electrospun fibers. Laser scanning confocal microscopy, LSCM, (Zeiss LSM 510 NLO) was used to observe the location of the heparin-bound dye. A fluorescence excitation wavelength of 747 nm and a KP685 filter were employed for these experiments. An oil immersion type Plan-Neofluor 40×/1.3 oil DIC (differential interference contrast) was used to obtain images in FIGS. 4, 5, 9, 10, 12 and a Plan-Apochromat 63×/1.4 oil Ph3 (phase-contrast—the 3 denotes a specific condenser lens used) was used to collect the image in FIG. 7. A z-series was made for each sample at 0.8 or 1.0 µm sections.

Toluidine Blue Assays

To determine the amount of heparin in the electrospun fibers, a common colorimetric method employing toluidine blue was used. A toluidine blue solution was made by dissolving 25 mg toluidine blue (Sigma Aldrich, St. Louis, Mo.) and 1 g NaCl in 0.49 mL of 37% HCl and 50 mL of water. The toluidine blue that reacts with the heparin is removed from solution via extraction with hexanes (5 mL). The amount of unextracted toluidine blue remaining in the aqueous solution was determined by measuring the absorbance at 631 nm and, by difference, provides an estimate of the heparin concentration.

Samples were prepared by adding 1 mg of electrospun fibers to a solution consisting of 0.8 mL of toluidine blue solution and 2.5 mL of 0.1% NaCl. The test tube was vortexed and 5 mL of hexanes was added with additional vortexing. After 10 minutes, 120 µL of the toluidine blue solution was transferred into a cuvette and absorbance measurements were taken. The control samples consisted of 1 mg of either PEO or PLGA fibers, without heparin, to adjust for the amount of toluidine blue that may adhere to the fibers. Measurements were performed in duplicate.

Growth Factor Binding

Growth factor binding assays were employed to determine if the LMWH and PEG-LMWH electrospun fibers were able to bind bFGF. All growth factor experiments were completed at room temperature in 24-well polystyrene assay plates (Corning Inc., Corning, N.Y.), blocked with 3% bovine serum albumin (BSA) (Sigma Aldrich, St. Louis, Mo.) in phosphate buffered saline (PBS) (Invitrogen, Carlsbad, Calif.). All electrospun samples were washed with PBS at room temperature. Samples were placed in the wells and incubated with 500 µL of a solution of basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, Minn., 10 ng/mL in PBS) for 2 h. After seven washes with 0.05 wt % Tween 20 (PBS-T), 500 µL of a solution of bFGF antibody was added (R&D Systems, Minneapolis, Minn., 2 µg/mL in PBS) and incubated for 2 h. The samples were washed seven more times with PBS-T. The samples were then incubated for 30 min. in a 500 µL solution consisting of 1 mL horseradish peroxidase conjugated NeutrAvidin (Pierce, Rockford, Ill., 1 mg/mL), 8 mL PBS, and 2 mL SuperBlock™ Blocking Buffer (Pierce, Rockford, Ill.). The fibers were then washed seven times with PBS-T and incubated in 500 µL of TMB substrate solution (Pierce, Rockford, Ill.) for 10-12 mins. Absorbance at 450 nm was measured. All absorbance values were normalized with respect to a well treated with bFGF and antibody, as explained previously, but without any electrospun fibers. The growth factor binding assay was completed three times and the average values are reported.
Results

Electrospinning Physical Mixtures (1:4) of LMWH and PEO

A 1:4 LMWH to PEO mixture (25 mg LMWH and 100 mg PEO) was dissolved in water and electrospun. The fibers resembled typical electrospun PEO fibers with an approximate diameter of 100-400 nm and were slightly beaded. The presence of microspheres with an average diameter of 1-5 µm was also observed. Two control solutions were made. One control consisted of a 10 wt % PEO/H₂O and the other a 10 wt % LMWH/H₂O. The 10 wt % PEO/H₂O solution electrospun to form PEO fibers with a diameter of 100-400 nm. The fibers were circular in their shape and contained beads (200-600 nm in diameter) but no microspheres were observed. The 10 wt % LMWH/H₂O control did not form fibers or microspheres, which is almost certainly due to the lack of entanglements in the negatively charged LMWH (3,000 g/mol). Attempts to electrospin higher MW heparin (12,000 g/mol) were not successful.

Attempts to electrospin higher MW heparin (12,000 g/mol) were not successful. Since no sulfur or sodium was detected, the results suggest that either the fiber section of the matrix consists of primarily PEO or that the concentration of sulfur is too low for detection via EDX. Generally, the lower limit of detection for EDX is approximately 1%, so it is possible that the LMWH may be incorporated into the fibers but at a concentration much lower than 1%. The beaded section of the matrix was found to contain 61% C, 13% O, 26% Al, and 0.3% S. The detection of the sulfur indicates that LMWH is present, at low concentrations, in the beaded areas of the electrospun mat. The microspheres were shown to contain 73% C, 20% O, 4% Al, 1.2% S, and 1.4% Na. The presence of sulfur and sodium imply that LMWH is present in the microspheres at higher concentrations than found in the beaded sections of the matrix. The EDX results are consistent with the expected composition of the fibers, and the varying amount of sulfur in the different regions suggests that LMWH is not uniformly incorporated into the fibers via simple mixing.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C (%)</th>
<th>O (%)</th>
<th>Al (%)</th>
<th>S (%)</th>
<th>Na (%)</th>
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<tr>
<td>PEO Fibers</td>
<td>46 ± 4.7</td>
<td>5.0 ± 1.15</td>
<td>49 ± 14.2</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>LMWH Film</td>
<td>53 ± 3.63</td>
<td>40 ± 2.16</td>
<td>ND</td>
<td>17 ± 1.41</td>
<td>18 ± 2.50</td>
</tr>
<tr>
<td>1:4 LMWH:PEO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>fiber section:</td>
<td>42 ± 9.02</td>
<td>5.0 ± 0.577</td>
<td>59 ± 18.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>bead section:</td>
<td>61 ± 2.65</td>
<td>13 ± 2.31</td>
<td>26 ± 4.51</td>
<td>0.3 ± 0.025</td>
<td>ND</td>
</tr>
<tr>
<td>microsphere section:</td>
<td>73 ± 2.52</td>
<td>20 ± 5.13</td>
<td>4.0 ± 1.66</td>
<td>1.2 ± 0.48</td>
<td>1.4 ± 0.13</td>
</tr>
</tbody>
</table>

*ND = not detected

Electrospinning Dye-Labeled LMWH with PEO

To visually map the location of the LMWH throughout the electrospun mat, a fluorescent dye, Alexa Fluor 3508, was covalently attached to LMWH, and the fibers were analyzed via LSCM; observation of fluorescence permits direct observation of the presence and location of LMWH throughout the electrospun matrix. PEO (10 wt %) was mixed with Alexa Fluor 3508-labeled LMWH (0.2 wt %) and electrospun. FIG. 1 shows resulting fibers with diameters of approximately 100-200 nm, with a few very large beads approximately 6 µm in diameter.

The fibers were then analyzed via LSCM to determine if the LMWH-dye molecules were incorporated into the matrix and to determine their distribution in the matrix. Electrospun PEO fibers were used as a control; results for the control are shown in FIG. 2. FIG. 2a shows data in reflection mode and confirms that fibers were present on the glass coverslip. FIG. 2b shows two small areas of fluorescence that are likely impurities in the PEO or dust in the sample, but confirms the complete lack of background fluorescence in the PEO fibers.

FIG. 3 presents LSCM data for electrospun fibers of the LMWH-dye/PEO mixture. The results demonstrate the fluorescence of the fibers, indicating the presence of LMWH. In contrast to SEM and EDX results, the LSCM results also suggest that the LMWH-dye is present not only in the beaded sections of the matrix, but also in the fiber regions. FIG. 3 shows the fluorescence of the fibers due to LMWH-dye incorporation. Z-stack images were also taken (not shown) to ensure that the LMWH-dye is present throughout the entire depth of the matrix and is not localized in any one area. Due to the LSCM limit of resolution, approximately 200 nm, the exact location of the heparin within each individual fiber is unknown. However, all LSCM images indicate that LMWH is present throughout the depths of the electrospun matrix, suggesting a fairly uniform dispersion of LMWH. Measurement of LMWH via toluidine blue assay confirmed the presence of approximately 4.3 µg (±0.923) of LMWH per mg of electrospun sample. A direct comparison cannot be made between the amount of LMWH in this sample and the amount in the physical mixtures of LMWH and PEO due to differing amounts of LMWH in the initial solutions. Nevertheless,
Incorporation of PEG-LMWH-dye in PEO for Electrospinning

LMWH was covalently attached to the termini of a 4-arm star PEO and evaluated for its incorporation into an electrospun matrix. PEG-LMWH-dye (0.2 wt %) was mixed with PEO (10 wt %) in water. The electrospun fibers from this solution were analyzed via FESEM, with results shown in FIG. 6. As is shown in FIG. 6, the resulting fibers were 100-300 nm in diameter with the presence of microspheres (1-3 μm). The morphology of the PEG-LMWH fibers was very similar to those produced from the earlier samples with free LMWH. In both electrospun mats, fibers are in the nanometer size range and the onset of bead formation is observed by the roughness of the fibers (see FIGS. 1 and 4).

The fiber matrix was then analyzed via LSCM to confirm that the PEG-LMWH-dye molecule was incorporated in the electrospun fibers; representative results are shown in FIG. 5. Similar to the results in FIG. 3, the data shown here also indicated that the fibers are evenly fluorescent across the matrix, showing that the PEG-LMWH was evenly distributed in the PEO fiber matrix. These fibers were found to contain 3.5 μg (+/-0.397) of heparin per mg of electrospun fibers. These results indicate a slight improvement in LMWH incorporation in comparison to the LMWH/PEO fibers, and may suggest that PEG-LMWH permits the incorporation of slightly more heparin than in the fibers produced using free heparin.

Changing the Matrix Polymer

Multiple polymers could be used as the matrix polymer. PLGA was chosen as an alternate carrier polymer for the LMWH systems due to the success of PLGA in biomaterial applications. PLGA/DMF (45 wt %) was electrospun with LMWH-dye (0.2 wt %) and also with PEG-LMWH-dye (0.2 wt %). Due to differences in molecular weight and viscosity, the concentration of carrier polymer is different than those used for PEO electrospinning, but all other processing conditions remained the same. The electrospun PLGA fibers are approximately 400-800 nm in diameter, (FIG. 6a). SEM revealed that fiber morphology was not affected by the incorporation of LMWH-dye or PEG-LMWH-dye, (FIGS. 6b and 6c).

LSCM showed that the PLGA electrospun mat, shown in FIG. 7a under transmitted light, does not fluoresce to any significant extent. As encountered previously with PEO fibers, there is a small area of fluorescence in FIG. 7b, which is likely due to polymer impurities or dust on the sample. The images shown in FIG. 8 reveal that both LMWH-dye and PEG-LMWH-dye were incorporated into the PLGA matrix and z-stack LSCM images confirm that the heparin is present throughout the depths of the matrices. The toluidine blue assay indicated that there is approximately 8.41 μg (+/-0.352) of LMWH present per mg of electrospun LMWH/PLGA sample. The PEG-LMWH/PLGA sample contained 9.50 μg (+/-0.382) of LMWH per mg of electrospun fibers. The results of the FESEM, LSCM, and the toluidine blue assays suggest that the carrier polymer can be changed without affecting the incorporation of heparin or fiber morphology.

Example 2

Growth Factor Release Assays of Electrospun Fibers

An ELISA format was employed to characterize the binding of bFGF in a series of samples: a blank well, PLGA fibers alone, LMWH/PLGA fibers, and PEG-LMWHP/PLGA fibers. Growth factor release studies were performed using a Quantikine Human FGF Basic Kit (R&D Systems, Minneapolis, Minn.). Incubation of the growth factor was completed using a 24-well polystyrene plate (Corning Inc., Corning, N.Y.) and transwell membranes (Fisher Scientific, Atlanta, Ga., 8 μm pore size) were used to secure the fibers during the release study. The plate, membranes, and fibers were blocked with 3% BSA (Sigma Aldrich, St. Louis, Mo.) in PBS (Invitrogen, Carlsbad, Calif.) overnight. All samples were washed three times with PBS at room temperature and then incubated with 4pL bFGF (R&D Systems, Minneapolis, Minn., 2.5 μg/mL in PBS) for 2 h at 4° C. The fibers were then transferred to the transwell membrane and 1 mL of PBS was added and incubated at 4° C., unless otherwise indicated, for a specific amount of time. The wells were replenished with fresh PBS after each aliquot was removed. The amount of bFGF present in the matrix was determined via use of a standard spectrophotometric immunochemical assay following the protocols of the manufacturer (R&D Systems, Minneapolis, Minn.). Small variations of the protocol could be necessary depending on the composition of the matrix.

The optical density measurements, at a wavelength of 450 nm, resulted in the following absorbance values relative to the control (increasing absorbance values correlate with increasing growth factor binding): PLGA fibers 1.59 (+/-0.055), LMWH/PLGA fibers 2.46 (+/-0.146), and PEG-LMWH/PLGA fibers 3.59 (+/-0.106). FIG. 9 shows that there are significant differences in bFGF binding between the LMWH/PLGA and PEG-LMWH/PLGA fibers, despite the fact that the toluidine blue assays show that the two matrices contain similar amounts of LMWH after electrospinning. Since both the LMWH and PEG-LMWH samples began with similar amounts of heparin but did not exhibit the same level of bFGF binding, these data suggest that the PEG-LMWH may be retained in the electrospun fibers more effectively than LMWH alone. FESEM characterization of the electrospun membranes after the growth factor binding assay revealed that the PLGA fibers exhibited no visible signs of degradation during the time frame of the growth factor binding study (FIGS. 10, parts a and b). This observation corroborates the fact that differences in growth factor binding likely result from differential LMWH retention rather than from variations in fiber degradation between samples. These electrospun membranes were also incubated in PBS for an additional 1 day, 3 days, and 14 days to determine if any fiber degradation occurred (FIG. 10, parts c-h). These SEM images show that there are no visible signs of fiber degradation for the time period studied in this investigation.

Characterization of the fibers after completion of the growth factor binding assays was conducted via multiphoton microscopy. FIG. 11, parts a and b, shows the LMWH retention within the electrospun fibers after the growth factor assay, or 6 h of PBS incubation. After only 6 h in PBS, slight differences in the amount of LMWH within the fibers were observed via multiphoton microscopy; these differences were much more pronounced with an additional 24 h incubation in PBS (FIG. 11, parts c and d). In FIG. 11, parts e-h, the multiphoton images are shown in fluorescence and reflection mode. This was necessary due to the small amount of fluorescence in the samples in order to prove that fibers are present. In FIG. 11, parts g and h, the most visible differences are observed. After 14 days incubation in PBS, the PEG-LMWH-dye fibers still exhibit a moderate level of fluorescence in comparison to the LMWH-dye fibers, which show minimal signs of fluorescence. The differences in the amount of LMWH retained in the fibers were also observed through-
bromodeoxyuridine (BrdU) incorporation (Cell Proliferation Branes were then placed in 24-well tissue culture dishes containing penicillin (100 U/mL) and streptomycin sulfate (100 µg/mL) for 2 h. The membranes were then washed 2-3 times with sterile PBS. All membranes were incubated in bFGF (15 ng/membrane) prior to the cell proliferation assay. The membranes were then placed in 24-well tissue culture dishes containing 1 % (v/v) FCS at 37° C. in an atmosphere of 95% air and 5% CO2. Culture medium containing 1% (v/v) FCS was changed every 30 min for 4 h. The scaffolds were then transferred to a solid phase peptide synthesis with Fmoc-protection using a PTI PS3 peptide synthesizer (Protein Technologies Inc, Tucson, Ariz.) and purified via preparative-scale reverse-phase HPLC. The PFZIP sequence CGGRMKOLEDVKKKLLKKNYHLENEVARLKKLVG is based on a GCN4 coiled coil and mimics the heparin-binding domain of human CRPKAKAKAKAKDQTK, respectively. Any heparin-binding peptide of sufficient affinity and appropriately slow dissociation (from heparin) would be equally applicable for use in these hydrogels. Peptides isolated as single fractions via HPLC were characterized via MALDI and ESI mass spectrometry. The mass of the purified HBPs was confirmed via HPLC and LC-ESI-MS/MS analysis. All materials were obtained and purified as described in Example 1.

Methods

Synthesis of PEG-LMW Conjugates

The synthesis of the PEG-LMW conjugate exploited in hydrogel assembly was conducted via Michael-type addition reactions between maleimide functionalized heparin and thiol-terminated four-arm star PEG, as described in Example 1. The degree of LMWH functionalization in PEG-LMW was determined to be 81% via 1H NMR spectroscopy. Synthesis of PEG-LMW-Alexa Fluor 350 was conducted via similar protocols as previously described in the art. Synthesis of PEG-LMW/PLGA electrospun mats was conducted via a similar setup as previously described in the art. Functionalized Cross-linked Matrices

Example 4

Materials

All materials were obtained and purified as described in Example 1.

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Methods

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Characterization

Peptides, PEG-LMWH, and PEG-HBPs were characterized via H NMR spectroscopy and MALDI-TOF Mass Spectrometry to determine purity, composition, and degree of functionalization.

Circular Dichroic Spectroscopy (CD)

CD spectra were measured at 5° C. to 75° C. on a J-810 spectropolarimeter (Jasco Inc., Easton, Md.) equipped with a Jasco PTC-424S temperature controller. Samples were equilibrated at the desired temperature for 30 minutes prior to data collection; equilibration was indicated by the absence of further changes in the CD signal at longer equilibration times. All CD spectra were taken in a 1 mm pathlength quartz cuvette, at wavelengths from 200 to 250 nm. Data points were recorded at every nanometer with a 4.0 s response time. The concentrations of peptide samples were determined via amino acid analysis for calculation of mean residue ellipticities.

Heparin Affinity Measurements

Heparin-Sepharose affinity liquid chromatography was performed on an ÄKTA Explorer FPLC equipped with a HiTrap Heparin HP column (5 ml, 10 mg heparin/ml gel, Amersham Biosciences Corp, Piscataway, N.J.). Surface Plasmon Resonance (SPR) SPR sensorgrams were recorded on a Biosero 3000 instrument (Biosero Inc, Piscataway, N.J.), employing a high-capacity LWMH surface created on a commercially available streptavidin-coated chip via treatment with biotinylated LWMH.

Bulk Rheology Experiments

Bulk rheology experiments of the PEG-LMWH/PEG-HBP hydrogels were conducted at 25 or 37° C. on a stress-controlled rheometer (MCR 500, Paar Physica, Anton-Paar, Ashland, Va.) with a 25.0 mm diameter parallel plate geometry and a 1 mm gap distance. Strain sweeps were performed on samples from 0.001% to a maximum strain of 100% to determine the limit of the linear viscoelastic regime (LVE). At a constant strain of 0.1%, chosen from the LVE, frequency sweeps ranging from 0.1 Hz to 100 Hz were conducted. Shear recovery experiments were also performed for the PEG-PF4ZIP-containing hydrogels, and prior to these experiments, a baseline oscillation at a constant strain of 0.1% and a constant frequency of 10 Hz was performed, followed by a high-speed rotation with a 1000 s-1 shear rate for 10 s. The recovery was then measured with a time sweep experiment at a constant strain of 0.1% and a constant frequency of 10 Hz.

Fluorimetry Experiments

Fluorimetry experiments to measure the concentration of PEG-LMWH-Alexa Fluor 350 (in eroded solutions of PEG-PF4ZIP-containing hydrogels) were performed using a FluorMax-3 spectrofluorometer (Jobin Yvon Inc, Edison, N.J.) with a xenon arc lamp illuminator. Erosion of PEG-LMWH from other PEG-HBP-containing hydrogels was determined gravimetrically. 45 µL of each sample were loaded into a cuvette and excited at 346 nm, the excitation spectra peak value of Alexa Fluor 350. The emission spectra peak value at 442 nm was recorded. Solutions of PEG-LMWH-Alexa Fluor 350 in PBS, with concentrations from 5.42x10-4 wt % to 4.23x10-6 wt %, were employed for generation of the standard curve. For erosion profile experiments, the PEG-LMWH-Alexa Fluor 350 was premixed with the PEG-LMWH at a weight ratio of 1:158. The fluorescence intensities of the PEG-LMWH-Alexa Fluor 350 in the eroded PEG-LMWH were then measured and permitted calculation of the percentage of PEG-LMWH eroded from the hydrogels in hydrogel erosion experiments.

Assembly of PEG-LMWH/PEG-HBP (and PEG-LMWH/FG) Hydrogels

The PEG-LMWH/PEG-HBP hydrogels were formed by the interaction between LMWH and PF4ZIP. 5 wt% solutions of the PEG-HBP in PBS were added directly to 2.5 wt% solutions of the PEG-LMWH in PBS, to afford hydrogel networks at a final composition of 2.5 wt% total polymer. The solutions were mixed in ligand mole ratios of 9:0.5, 8:1, and 7.5:2.5 (LMWH:PF4ZIP dimer); other ligand mole ratios are also useful for hydrogel assembly. For VEGF-crosslinked hydrogels, an 8 wt% solution of PEG-LMWH in PBS is mixed with varying amounts of solid VEGF (0.1-20 µg) to afford a self-supporting gel upon sufficient addition of VEGF. After mixing, the hydrogels were stored at 4° C. The stored hydrogels were equilibrated to room temperature prior to rheological characterization (typically several minutes).

bFGF Release from Hydrogel Experiments

bFGF release and hydrogel erosion experiments were performed at 4° C. in 24-well polystyrene assay plates (Corning Inc., Corning, N.Y.), blocked with PBS containing 5% BSA and 0.05% Tween 20 (Sigma, St. Louis, Mo.). The wells were aspirated and washed 4 times with 0.05% Tween 20 in PBS before loading samples. Afterward, 200 µL hydrogel was placed on the bottom of each well and 2.8 mL PBS was added carefully over each gel. 1 mL PBS was taken out of the well at 1, 2, 6 and 24 h and then each following day, followed by replacement with 1 mL fresh PBS. The amount of bFGF in each sample was measured with a bFGF Quantikine kit (R&D Systems, Minneapolis, Minn.). The hydrogel erosion kinetics were conducted on the same samples used for bFGF release experiments.

Results

Oligomerization of PF4ZIP

Our initial studies were aimed at determining the oligomerization properties of the coiled-coil PF4ZIP, as such information is relevant to the subsequent use of the PF4ZIP in hydrogel formation. No such characterization was necessary for the other HBPs, as they do not form higher-order assemblies. Circular dichroic spectroscopy studies were conducted to assess the folding of the PF4ZIP under various conditions. Solutions of PF4ZIP dissolved in PBS were scanned from 250 nm to 200 nm at varying temperatures between 5° C. and 75° C. The CD spectra of PF4ZIP at a variety of temperatures show an isodichroic point at 203 nm, validating the existence of a two-state equilibrium. At temperatures below 55° C., the spectra observed exhibit two minima at 208 nm and 222 nm, demonstrating that PF4ZIP exists in the coiled-coil state under conditions of interest for hydrogel applications. As seen by the appearance of a single minimum at 203 nm in the data collected at elevated temperatures, the structure of PF4ZIP reversibly changes to random coil at elevated temperature. Maximum mean residue ellipticity values at 222 nm (MRE222) were measured to be -30,470 deg cm2 dmol-1 for the PF4ZIP at 15° C., consistent with reported values ranging from -25,000 to -35,000 deg cm2 dmol-1 for various GCN4-mimic peptides in PBS at 15° C.
The conformation of PF4ZIP at various concentrations was also monitored via CD. At all concentrations, the spectra observed exhibit two minima at 208 nm and 222 nm, demonstrating the helical character of PF4ZIP under these conditions. The measured MRE for a solution of 56 μM PF4ZIP in PBS is ~24,580 deg cm² dmol⁻¹, which is consistent with the value reported for 40-60 μM PF4ZIP in 3 mM sodium citrate buffer (~27,000 deg cm² dmol⁻¹). The difference between the measured ellipticity and the ellipticity of the unfolded monomer is related to concentration and can be fit to determine the Kd of the dimerization, via standard fitting procedures well-described in the literature. The calculated equilibrium constant of unfolding, Kd, is 3.48±0.03 μM. Solutions of PF4ZIP attached to star-PEG were also studied via CD, to ensure that conjugation to PEG did not significantly alter the association of the PF4ZIP coiled-coils. As shown in Fig. 17(a), the spectra of PEG-PF4ZIP are essentially identical to the spectra obtained for the isolated PF4ZIP. Small differences in MRE values likely arise from uncertainties in the precise peptide concentrations for the PEG-PF4ZIP samples. The Kd value derived from the best fit of the concentration dependence of (Fig. 17(b)) for PEG-PF4ZIP is 0.93 μM. The PF4ZIP concentrations during NMR characterization and hydrogel formation and characterization are always much greater than these measured Kd values (e.g., 200 μM and higher), suggesting that PF4ZIP will exist as a fully folded dimer under those conditions.

Heparin-binding Affinity

Our previous investigations of PEG-LMWH/PEG-HBP hydrogels suggested that the viscoelastic properties of the noncovalently assembled hydrogels were related to the heparin-binding kinetics and affinities of the peptides (from anti-thrombin III (ATIII) or heparin interacting protein (HIP)). Heparin-Sepharose affinity chromatography and SPR experiments were therefore used to compare the heparin-binding affinity of the various HBP samples. Results of these experiments are summarized in Table 2.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Kd (M⁻¹ s⁻¹)</th>
<th>kₐ (s⁻¹)</th>
<th>Kₐ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATIII²</td>
<td>594 ± 2</td>
<td>1.56 ± 0.03 x 10⁶</td>
<td>2.00 ± 0.3 x 10⁻³</td>
</tr>
<tr>
<td>HIP⁵</td>
<td>687 ± 1</td>
<td>1.10 ± 0.08 x 10⁶</td>
<td>4.04 ± 0.02 x 10⁻³</td>
</tr>
<tr>
<td>PF4ZIP</td>
<td>962 ± 10</td>
<td>2.24 ± 0.05 x 10⁶</td>
<td>2.56 ± 0.10 x 10⁻³</td>
</tr>
</tbody>
</table>

¹Data from previously reported studies (N. Yamaguchi, B. S. Chae, L. Zhang, K. L. Kiick, E. M. Furst, Rheological characterization of polysaccharide-poly(ethylene glycol) star copolymer hydrogels, Biomacromolecules 6 (4) (2005) 1931-1940)

For heparin affinity chromatography experiments, HBPs were loaded onto a heparin-Sepharose column, and then eluted with buffer of increasing NaCl concentration. The concentration of NaCl required for peptide elution is used as an indication of the affinity of the peptide for heparin. The elution of the peptides from the heparin-Sepharose column required a salt concentration of 962±10 mM for the PF4ZIP peptide. The salt concentration required to elute PF4ZIP (962±10 mM) is higher than that measured for the ATIII peptide (594±2 mM) and for the HIP peptide (687±1 mM), indicating that PF4ZIP has higher heparin-binding affinity than the ATIII and HIP peptides and should therefore be competent for hydrogel assembly. These results are consistent with previous reports indicating that similarly high concentrations of NaCl were required to elute 125I-labelled PF4ZIP bound to a heparin-Sepharose column in 0.02 M Tris-HCl buffer pH 7.0 containing 0.05 M NaCl.

In SPR experiments, the interaction of the peptides with a LMWH-modified SA chip surface was determined under conditions described in the experimental section. Neither nonspecific binding of heparin-binding proteins to SA chips nor chip instability was observed in any of the SPR measurements. In addition, no mass transport limitation or linked reactions were indicated. The measured association of the PF4ZIP with LMWH proceeds with a rate constant of 2.24±0.05 x 10⁶ M⁻¹ s⁻¹, while the dissociation proceeds with a rate constant of 2.56±0.10 x 10⁻³ s⁻¹, yielding a KD of approximately 1.15±0.03 x 10⁻² μM. These faster association kinetics, relative to those reported for the ATIII and HIP (Table 2) (see also N. Yamaguchi, B. S. Chae, L. Zhang, K. L. Kiick, E. M. Furst, Rheological characterization of polysaccharide-poly(ethylene glycol) star copolymer hydrogels, Biomacromolecules 6 (4) (2005) 1931-1940) are the primary cause of the lower measured KD values for PF4ZIP-LMWH binding, and the trend of the dissociation constants is consistent with the chromatography results. The more rapid association rate, coupled with the similar dissociation rate, of PF4ZIP versus the other HBPs, points to the successful formation of hydrogels between PEG-PF4ZIP and PEG-LMWH. The measured Kd of the binding between PF4ZIP and LMWH is lower than the measured KD of dissociation of the coiled-coil obtained from CD experiments, which may suggest that the LMWH-functionalized surface facilitates coiled-coil formation. Nevertheless, the equilibrium constants of dissociation determined via both methods are substantially lower than the concentrations employed during hydrogel formation experiments (see below).

Temperature Dependence of PF4ZIP Binding

The heparin-binding kinetics between LMWH and PF4ZIP were monitored via SPR at several temperatures between 5° C. and 37° C., to determine potential changes in binding
kinetics that might alter hydrogel properties under physiological conditions. Data from these experiments are shown in Table 3.

### Table 3

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>$k_{s}$ (M⁻¹-s⁻¹)</th>
<th>$k_{w}$ (s⁻¹)</th>
<th>$K_{s}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.56 ± 0.01 x 10⁶</td>
<td>3.74 ± 0.17 x 10⁻³</td>
<td>2.41 ± 0.10 x 10⁻⁸</td>
</tr>
<tr>
<td>15</td>
<td>1.90 ± 0.04 x 10⁶</td>
<td>1.67 ± 0.03 x 10⁻³</td>
<td>8.82 ± 0.02 x 10⁻⁸</td>
</tr>
<tr>
<td>25</td>
<td>2.24 ± 0.05 x 10⁶</td>
<td>2.56 ± 10⁻³</td>
<td>1.15 ± 0.03 x 10⁻⁸</td>
</tr>
<tr>
<td>37</td>
<td>5.11 ± 0.02 x 10⁵</td>
<td>2.44 ± 6.08 x 10⁻³</td>
<td>4.78 ± 0.14 x 10⁻⁹</td>
</tr>
</tbody>
</table>

The measured on and off rates fluctuate only slightly throughout the temperature range, suggesting that noncovalently assembled hydrogels based on PEG-ZIP and LMWH interactions should exhibit consistent mechanical properties at these temperatures.

**PEG-LMWH/PEG-HBP Hydrogel Formation**

Via 1H NMR spectroscopy, functionalization of the star PEG with HBPs was determined to be at least 59%, indicating that PEG-HPBs meet the functional requirement (at least one binding partner exhibits f>2) for crosslinking a hydrogel. Before addition of PEG-PF4ZIP, the 2.5 wt % of the binding partner exhibits f>2) for crosslinking a hydrogel than the loss modulus $G''(w)$ over all measured frequencies.

Hydrogels were characterized via theological frequency formation, although some oligomers of the PEG-PF4ZIP bio-terminated star polymers via intermolecular coiled-coil for- that there was no significant crosslinking of the peptide-water-like viscosity of the PEG-PF4ZIP solution indicated that PEG-LMWH was uniform and translucent. In addition, the PEG-LMWH/PEG-PF4ZIP hydrogels were also investigated at physiological temperature. The mechanical properties of the hydrogels with molar ratios of LMWH to PF4ZIP of 9:0.5 and 7.5:2.5 were examined. The mechanical properties of the hydrogels are initially estab-

The frequency sweep data for the hydrogel (LMWH:PF4ZIP=9:0.5) are presented in FIG. 20. For $G'(w=0.1 Hz)$ plotted as a function of the ratio of HBP to LMWH in the samples, with moduli normalized by the initial storage modulus of PEG-LMWH. The plateau in the modulus with increasing PF4ZIP or HIP concentration may imply that the HBP-binding sites on LMWH have been saturated. The increase in the elastic modulus upon addition of PEG-PF4ZIP for the PEG-LMWH/PEG-PF4ZIP hydrogels is lower than that observed with addition of PEG-HBP for the PEG-LMWH/PEG-ATIII or the PEG-LMWH/PEG-HIP hydrogels. Literature reports suggest that at least 4 tetrasaccharides (16 units) are required for efficient heparin-PF4 protein interactions, while HIP- and ATIII-binding sites are believed to be similar pentasaccharides in heparin. The fewer PF4-binding sites on LMWH could lead to the lower storage modulus plateau value and lower slope in FIG. 20 for the PEG-LMWH/PEG-PF4ZIP hydrogels than that observed for the PEG-LMWH/PEG-ATIII or the PEG-LMWH/PEG-HIP hydrogels. These results indicate two things. First, the mechanical properties of the hydrogels are initially estab-

5. A similar dependence of rheological properties on the concentration of heparin-binding crosslinker was observed for PEG-LMWH crosslinked with VEGF. Laser tweezer micro rheology experiments, in this case, confirmed the elastic behavior of the hydrogels at low and moderate frequencies, and also demonstrated that the development of elastic properties of the network are dependent on the concentration of VEGF present in the network. Micro rheology character-

6. ization was conducted on 4 wt % hydrogels in order to reduce the elastic modulus sufficiently for measurement. 8 wt % hydrogels provide improved mechanical properties, although a variety of concentrations could be used depending on the desired application.

The rheological properties of the PEG-LMWH/PEG-PF4ZIP hydrogels were also investigated at physiological temperature. The mechanical properties of the hydrogels with molar ratios of LMWH to PF4ZIP of 9:0.5 and 7.5:2.5 were examined. The frequency sweep data for the hydrogel (LMWH:PF4ZIP=9:0.5) are presented in FIG. 21. The moduli at 25° C. or 37° C. are essentially identical, with an identical frequency dependence, although the gels at 37° C. exhibit slightly lower loss moduli at higher frequencies. The materials are therefore also elastic hydrogels at 37° C., in
corroboration of the SPR studies. This lack of temperature dependence is also observed in the hydrogel with higher PF4ZIP content, reflecting the temperature insensitivity of both the PF4-LMWH interactions and the LMWH-LMWH interactions. The shear recovery data for the PEG-LMWH/PEG-PF4ZIP hydrogels are exhibited in FIG. 22. Both the extent and time-dependence of recovery were determined in these experiments. FIG. 22 indicates that after the hydrogels are subjected to a steady shear stress and recovery is observed, they essentially immediately recover their elastic properties, so only a slight reduction in modulus can be measured. In addition, the moduli at 25°C and 37°C are equal, with a matching kinetics of recovery. The data are consistent with previous rheological frequency sweep studies (FIG. 21). The immediate recovery and stability of the PEG-LMWH/PEG-PF4ZIP hydrogels even after subjected to high shear suggests their potential utility in injectable applications.

Growth Factor Release and Hydrogel Erosion

The release of growth factors from these hydrogels in vitro has also been investigated. Although covalently crosslinked, heparin-containing hydrogels have been shown to be useful for release of bFGF, physically crosslinked hydrogels could provide an alternative protein-delivery matrix without the need for potentially toxic crosslinking reagents.

The bFGF release and hydrogel erosion experiments were performed at 4°C in 24-well polystyrene assay plates. A 2.5 wt% solution of PEG-LMWH with bFGF was prepared via dissolving PEG-LMWH and bFGF in PBS at a final molar ratio of approximately 1:1,500,000 (bFGF to LMWH). Secondly, a 5 wt% solution of PEG-PF4ZIP (or other PEG-HBP) in PBS was added to the PEG-LMWH/bFGF at a ligand molar ratio of 8:1 (LMWH/PF4ZIP: bFGF). Other molar ratios can also be used, depending upon the desired rate of release of the growth factor and/or desired rate of erosion of the network. Assuming that all HBPs on the PEG-HBP termini bind LMWH, the molar ratio of bFGF to available LMWH in the PEG-LMWH/PEG-HBP hydrogels is approximately 1:1,300,000.

Data from studies of bFGF release from PEG-LMWH/PEG-HIP and PEG-LMWH/PEG-PF4ZIP hydrogels are summarized in FIG. 23. As shown in the figure, a burst release of bFGF ranging from 10 to 25% (depending on gel identity) is observed, followed by a slow release of bFGF over 10 days, to a maximum measured release ranging from approximately 20 to 35%. Such release profiles are of potential use in neovascularization, wound repair, and tissue engineering applications. As shown in FIG. 23, the bFGF is released slightly more quickly from the PEG-LMWH/PEG-HBP hydrogels than from the PEG-LMWH/PEG-HIP hydrogels, indicating that the rates of release of growth factor from these hydrogels are tunable based on hydrogel composition. The PEG-LMWH/PEG-HIP hydrogel demonstrates higher storage and loss moduli than the PEG-LMWH/PEG-PF4ZIP hydrogels. The observed burst release is similar in value to previously reported burst release in other heparinized hydrogel systems, and the linear release following the initial burst in those hydrogel systems has been successfully applied to stimulate cell proliferation.

Hydrogel erosion kinetics are also shown in FIG. 23. In the case of the PEG-LMWH/PEG-PF4ZIP hydrogels, PEG-LMWH-Alexa Fluor 350 was pre-mixed with PEG-LMWH at a weight ratio of 1:1.58 to facilitate tracking of the eroded PEG-LMWH via fluorimetry. The erosion profile of the PEG-LMWH/PEG-HIP hydrogels was determined gravimetrically in the transwell assay format in conjunction with the bFGF release experiments. As shown in the figure, a burst erosion of 10 to 30% is initially observed (depending on hydrogel composition), followed by a slow erosion to a maximum of 30 to 50% (again, depending on hydrogel composition). As is also indicated in this figure, the growth factor release is directly correlated to the erosion of the hydrogel, and can therefore be controlled by modulating hydrogel mechanical properties. Modulating the affinity of the growth factor to the hydrogel will also serve as an additional strategy to control release rates.

Growth factors released from hydrogels of these general compositions have also been shown to be bioactive, indicating the utility of these matrices in desired applications. For example, bFGF released from PEG-LMWH functionalized hydrogels (covalently crosslinked model systems) (procedure for covalent crosslinking is provided in N. Yamaguchi, K. L. Kiick, Polysaccharide-poly (ethylene glycol) star copolymers as scaffolds for the production of bioactive hydrogels, Biomacromolecules 6 (4) (2005) 1921-1930, which is incorporated herein by reference) has been shown, via MT assay, to increase proliferation of HUVECs (human umbilical vein endothelial cells) in a bFGF-dose-responsive manner. In these assays, the amount of yellow MTT oxidized to purple formazan is measured spectrophotometrically as a function of the amount of bFGF loaded in the hydrogel. Average increases in formazan salt formation ranged from 1.5 to 4 fold in the bFGF proliferation experiments. In another set of experiments, VEGF released from the PEG-LMWH/VEGF hydrogels has been shown to increase the rate of proliferation of porcine aortic endothelial (PAE) cells in DMEM media. Assays were conducted in a transwell assay format as described in N. Yamaguchi, K. L. Kiick, Polysaccharide-poly (ethylene glycol) star copolymers as scaffolds for the production of bioactive hydrogels, Biomacromolecules 6 (4) (2005) 1921-1930. Measured increases in the proliferation rate, assessed via cytometry, have ranged from 25-50%. These results complement and are similar to the results obtained in Example 3, which indicate the bioactivity of growth factor-loaded electrospray matrices. Similar bioactivity of any growth factor sequestered in these matrices is expected, based on these results, and on the general observation of growth factor activity preservation in heparinized matrices.

What is claimed is:

1. A crosslinked matrix comprising at least two compatibilizing polymer-LMWH conjugates, wherein the compatibilizing polymer comprises PEG, and at least one crosslinking compound wherein each compatibilizing polymer-LMWH conjugate is capable of assembly and comprises at least one compatibilizing polymer covalently bonded to at least one LMWH molecule, wherein each of the compatibilizing polymer-LMWH conjugates is partially nocovalently crosslinked with the same crosslinking compound, and wherein the crosslinking compound is an incorporated molecule comprising a heparin-binding growth factor or a heparin-binding compatibilizing polymer-peptide conjugate.

2. The crosslinked matrix of claim 1 wherein the crosslinking compound comprises a heparin-binding compatibilizing polymer-peptide conjugate.

3. The crosslinked matrix of claim 2 wherein the heparin-binding compatibilizing polymer-peptide conjugate comprises PF4ZIP, HIP, or ATIII.

4. The crosslinked matrix of claim 1 wherein the LMWH has an average molecular weight of about 10,000 daltons.

5. The crosslinked matrix of claim 1 wherein the compatibilizing polymer comprises four-arm star PEG.
6. The crosslinked matrix of claim 1 wherein the LMWH has an average molecular weight of about 3,000 daltons.

7. The crosslinked matrix of claim 1 wherein the incorporated molecule release is directly correlated to the erosion of the matrix.

8. The crosslinked matrix of claim 1 wherein the incorporated molecule release is directly correlated to cell-receptor interactions with the matrix.

9. The crosslinked matrix of claim 2 wherein the heparin-binding compatibilizing polymer-peptide conjugate is capable of assembly and comprises at least one compatibilizing polymer covalently bonded to at least two heparin-binding peptides.

10. A crosslinked matrix comprising at least three compatibilizing polymer-LMWH conjugates, wherein the compatibilizing polymer comprises PEG, at least one incorporated molecule comprising a heparin-binding growth factor and at least one heparin-binding compatibilizing polymer-peptide conjugate wherein each compatibilizing polymer-LMWH conjugate is capable of assembly and comprises at least one compatibilizing polymer covalently bonded to at least one LMWH molecule, wherein at least two of the compatibilizing polymer-LMWH conjugates are partially noncovalently crosslinked with the same incorporated molecule, wherein at least two of the compatibilizing polymer-LMWH conjugates are partially noncovalently crosslinked with the same heparin-binding compatibilizing polymer-peptide conjugate.

11. The crosslinked matrix of claim 1 wherein the crosslinking compound comprises a heparin-binding growth factor.

12. The crosslinked matrix of claims 10 or 11 wherein the growth factor comprises fibroblast growth factor, vascular endothelial growth factor, heparin-binding epidermal growth factor, transforming growth factor, acidic fibroblast growth factor or basic fibroblast growth factor.