Advanced Review

Heparin-based nanoparticles

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The combination of nanoparticles and biological molecules is of intense interest because of the synergistic properties offered by such newly synthesized composites. Heparin (HP), conjugated to nanomaterials, has recently been investigated for its chemical and biological properties. HP has a number of biological activities that can be enhanced when composited with nanoparticles. In addition, HP improves the biocompatibility of nanoparticles improving their performance in various biological applications. A variety of recent research combines HP and nanomaterials for a myriad of applications. HP has been conjugated to the surface of the nanoparticles, such as magnetic and metallic nanoparticles, or biodegradable and nondegradable synthetic polymers. HP has also been incorporated into the nanoparticles. There are numerous possibilities for material composites and chemistries that incorporate HP. This opens the door for novel applications ranging from improving anticoagulant activity, for anticancer and antitumor therapy, to tissue engineering and biosensors. This review examines the different possibilities of HP-based nanoparticle composites and their medicinal or biological applications. *WIREs Nanomed Nanobiotechnol* 2010 2 77–87

Nanoparticles have been used for thousands of years. They have been used for aesthetic, as well as curative purposes. Many materials have been used in nanotechnology, each having distinct properties for specific applications. The physical and biological properties of nanomaterials are unique and differ depending on their corresponding bulk material, making these entities an intense focus of recent studies. As nanoparticles are typically classified between 1 and 100 nm in size, which is in the range of biological molecules and much smaller than cells (1 – 10 m), they also have great potential in biological applications. The incorporation of biological molecules, such as proteins, DNA, bacteria, viruses, on or within nanoparticles has provided novel and enhanced activities with potential applications in therapeutics, biosensors, imaging, drug delivery, etc. These biomolecules also can passivate the surfaces of nanoparticles, improving their biocompatibility and allowing the nanoparticles to slip through undetected evading the natural immunological response. One class of molecules of interest that can cloak these foreign materials is a family of polysaccharides, called glycosaminoglycans (GAGs), which naturally cover the surface of all eukaryotic cells.

GAGs are negatively charged polysaccharides composed of repeating disaccharides units. GAGs are often located on the surface of cells or within the extracellular matrix (ECM). They are generally covalently linked to core proteins comprising larger glycoconjugates called proteoglycans (PGs)[1] and participate in a wide variety of biological events, which depend on their core structures, core proteins, and sulfation patterns. The structurally related heparin (HP) and heparan sulfates (HS) are one such specific family of GAGs. HP is located in the mast cells and its exact biological function is not clear, but it is thought to be involved in allergic and inflammatory responses.[2,3] HS is ubiquitous within animals, from *Caenorhabditis elegans* to humans, and is located on the membrane and within the ECM of virtually all tissues.[4] HP and HS are linear polysaccharides with a repeating disaccharide unit of 1,4-linked uronic acid [D-glucuronic (GlcA) or L-iduronic acid (IdoA)] and D-glucosamine (GlcN) residues. Both uronic acid and GlcN can contain sulfo groups at different positions of the pyranose ring including 2-*O*-sulfo substitution of the uronic acid residue and 2-*N*-, 3-*O*- and 6-*O*-sulfo substitution in the GlcN residue (Figure 1(a)). HP has an average molecular weight of ~12,000 Da, corresponding

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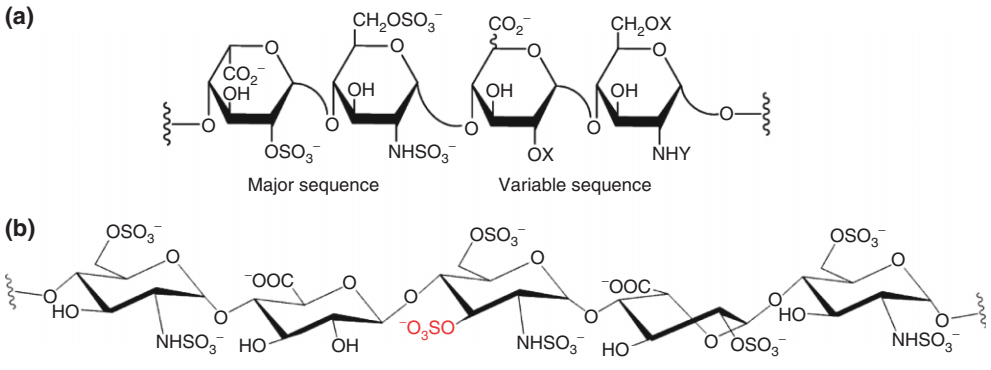

FIGURE 1 | Structure of heparin (HP). (a) Structure of HP major and minor variable sequences (X = sulfo or H, Y = sulfo, Ac, or H). (b) HP pentasaccharide Antithrombin III (ATIII) binding sequence. The red highlighted sulfo group at the C-3 position of the center glucosamine residue is critical for the interaction with ATIII.

to ~20 disaccharide units per chain, with a polydispersity of 5,000–40,000 Da. HP is more sulfated than HS, with an average of 2.7 sulfo groups per disaccharide compared with <1 sulfo groups per disaccharide for HS.[5] These sulfo groups, along with carboxyl groups, give HP an average charge of approximately −75, making it the most negatively charged natural product known.[2] The polydispersity of the chains and structural variations make HP and HS very heterogeneous, highly charged, polysaccharides.[6] Their heterogeneity allows them to interact with many different proteins to carry out various biological activities.

HP is most commonly known as an anticoagulant. It has been used as a drug since the 1930s, and is one of the few natural product therapeutics still in clinical use today.[3] Antithrombin III, a serine protease inhibitor, is able to recognize and bind to a specific pentasaccharide sequence within the HP chains (Figure 1(b)). This interaction causes a conformational change within the protein allowing it to inhibit thrombin and other serine proteases within the coagulation cascade.[2] The natural propensity for blood to clot protects the body from a significant loss of blood during injury. This activity, while beneficial in some instances, such as during surgical procedures or extracorporeal therapies, can be harmful in others. Blood clots when coming in contact with synthetic materials used for medical devices. These medical devices and treatments often activate the coagulation cascade causing clot formation. In these cases, the inhibition of the coagulation cascade is important.[2] The anticoagulant activity of HP has been widely studied; however, it also has been shown that HP exhibits other biological activities through its interaction with various proteins. HP has been shown to have antiviral activity, specifically inhibiting the replication of human immunodeficiency virus type 1.[7,8] It also can control tumor growth and inhibit angiogenesis, making it a candidate for cancer therapeutics.[9,10] It is also able to inhibit the complement cascade[11,12] and participates in the release of lipoprotein lipases.[13,14] HP has also been medicinally used for treatment of wounds in burn victims. It is hypothesized that HP can relieve pain associated with the burns, inhibit clotting and inflammation, and accelerate the healing process by promoting cellular epithelialization.[15,16] HP has diverse biological roles, giving it the potential to be exploited for therapeutic use to treat many different ailments and diseases.

Recently, research has begun to combine the useful biological activities of HP with the useful properties of nanomaterials. The combination of the two substances can provide synergistic improvements enhancing already existing properties and applications, and also create novel uses for these composites. Table 1 summarizes recent publications describing the combination of HP with nanomaterials. This review focuses on selected studies on heparinized nanoparticles and the potential therapeutic applications for these composites.

DRUG DELIVERY

HP and its derivative, low-molecular-weight HP (LMWHP), have been used for the prevention of thrombosis for over 60 and 20 years, respectively. HP is given parenterally to many patients, by *intravenous* or *subcutaneous* injection, which is often an invasive therapy. An oral HP would certainly be desirable, but HP is not efficiently absorbed through the gastrointestinal tract when taken orally and has a relatively short half-life.[39] This low bioavailability, explained by HP's high molecular weight and highly negative charge, results from its repulsion from the negatively charged mucosal layer and epithelial cells and its inability to cross membranes.[40] Therefore, methods have been investigated to increase both the bioavailability and the half-life of this important drug. One area being investigated is to use nanoparticles as carriers for HP. Various materials have been investigated for their biodegradability, biocompatibility,

TABLE 1 | Summary of Heparinized Nanomaterial and Their Potential Applications

Nanoparticle	Nanoparticle chemistry	Application	Reference
Synthetic polymer			
Poly(methyl methacrylate)	Self-assembled amphiphile	Drug delivery	Passirani[17,18]
Poly-ε-caprolactone	Electrostatic	Anticoagulant drug delivery	Jiao[19], Lamprecht[20]
Poly(lactic-*co*-glycolic acid)			
Eudragit			
Hydroxyapatite	Electrostatic layer-by-layer with poly(L-histidine)	Bone-engineering scaffolds	Rai[21]
Poly(alkylcyanoacrylate)	Self-assembled amphiphile	Oxygen carrier	Chauvierre[22–24]
Metal			
Gold nanoparticles	Reduction of metal salts with HP chains Amine on diaminopyridine moiety on HP interacts with surface	Biocompatibility, drug carrier	Guo[25] Huang[26]
Silver nanoparticles			Kemp[27]
Magnetic			
Iron oxide	Electrostatic layer-by-layer with poly(L-lysine)	Targeted drug carrier	Khurshid[28]
Biopolymer			
Deoxycholic acid–HP	Self-assembled amphiphile	Drug delivery for cancer treatment	Park[29,30]
Poly(L-lysine)–HP	Electrostatic	Tissue engineering	Na[31], Park[32,33]
Chitosan–HP	Ionic gelation	Anti-*Helicobacter pylori* therapy, drug delivery	Lin[34], Liu[35]
HP nanogels	Thiolated HP generated disulfide bonds	Apoptotic cell death for cancer treatment	Bae[36]
Core/shell			
poly(vinyl alcohol)/iron oxide	Aminotrimethoxysilane and 4,4-diphenyl diisocyanate linkers	Anticoagulant recycling	Liu[37]
Pluronic/HP	HP crosslinks activated poloxamer of [poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide)]	Drug carrier and imaging agent	Choi[38]

or other unique properties. Jiao et al. tested HP conjugated to both positively charged biodegradable, poly-ε-caprolactone and poly(lactic-*co*-glycolic acid) (PLGA), and nonbiodegradable polymethacrylates, Eudragit RS and RL, for oral bioavailability in rabbits.[19] HP was entrapped in these polymeric nanoparticles by a multiple emulsion process, with diameters ranging from 260 to 300 nm. The anticoagulant activity, of these nanoparticle composites, was evaluated to determine both their *in vitro* and *in vivo* efficacy. The authors showed that HP-loaded particles still retained their anticoagulant activity compared with orally administered free HP. They deduced that the HP is protected from degradation by the nanoparticles, and is slowly being released from the particles in an unaltered state.

Others have observed that nanoparticles increase the bioavailability of HP when administered orally, but little is known about why this occurs. Lamprecht et al. used a resonant mirror system and Caco-2 cells to investigate the mechanism on how HP nanoparticles interact with mucin and epithelial cells, respectively.[20] HP was immobilized to similar nanoparticles to those used by Jiao, a nonbiodegradable polymer, Eudragit RS, and the biodegradable PLGA. From adhesion studies Lamprecht et al.[20] deduced a two-part mechanism in which nanoparticles were able to first interact with the negatively charged mucosal layer on the epithelial cells. The

mucoadhesive properties of the nanoparticles exposed the HP drug to the epithelial barrier for a longer amount of time. Mucin, a highly negatively charged glycoprotein, could then displace HP on the particle's surface, thereby releasing the drug into the body. By investigating the mechanism of adhesion to explain the increased bioavailability, new methods can be developed that would allow more enhanced absorption of HP through oral administration, which could eliminate the need for repeated invasive injections.

Research has been performed on using the biological and chemical properties of HP to develop more efficient administration methods to decrease side effects associated with repeated HP injections, such as HP-induced thrombocytopenia, osteoporosis and alopecia. Chen and coworkers proposed using magnetic nanoparticles for the recycling of HP, by separating the nanoparticles in a magnetic field. HP was conjugated to an iron oxide shell surrounding a poly(vinyl alcohol) core, with retention of anticoagulant activity.[37] HP was successfully conjugated to the particles through covalent bonds by using aminotrimethoxysilane (ATMS) and 4,4-diphenyl diisocyanate (HMDI) as a spacer between the particle and HP chain (Figure 2). Different-sized linkers between the particles and the HP chains were tested for retention of anticoagulant activity. For shorter spacers, HMDI was directly conjugated through the hydroxyl group present on the iron oxide shell and one end of the isocyanate, while the other isocyanate was attached to the HP chain. For the longer spacers, silane from the ATMS reacts with the hydroxyl groups on the iron oxide shell, exposing the amine moiety to react with the isocyanate group of the HMDI. HP is then conjugated to the other end of the isocyanate group (Figure 2). Liu et al.[37] showed that HP had better anticoagulant ability when conjugated to the longer spacer arm because the longer spacer arm prevented steric hindrance allowing for a higher density of HP/particle. They proposed using magnetic nanoparticles for recycling of HP by separating the nanoparticles by a magnetic field from the blood before injection into the body. This would decrease side effects associated with repeated HP injections. The authors see this work as useful for blood transfusions where HP needs to be injected with the blood into the patient to prevent coagulation. Removing the HP before it gets into the body can decrease any allergic reactions that can often occur with such high dosages.

CANCER THERAPY

There is a myriad of materials that can be synthesized or fabricated into nanoparticles. Magnetic nanoparticles, especially iron oxide, have unique features and have been used in many different biomedical applications, such as magnetic resonance imaging, cell sorting, targeted drug delivery, and cancer treatments.[41] As previously discussed, iron oxide nanoparticles have been conjugated to HP for recycling anticoagulants. In their work, Khurshid et al. develop a method for coating HP and LMWHP on iron oxide nanoparticles.[28] Instead of using ATMS and HMDI as spacer arms for the covalent linkage of the HP to the particles, Khurshid et al. coated the iron oxide particles with a layer of positively charged poly(L-lysine) (PLL), HP was then attached to the PLL layer through electrostatic interactions. The average particle size for the LMWHP particle sample was $\sim 20 \pm 10$ nm using transmission electron microscopy analysis, while X-ray photoelectron spectroscopy, zeta potential, and Fourier transform infrared spectroscopy confirmed the absorption of PLL and the HP onto the particles. Magnetic measurements with a vibrating sample magnetometer showed a decrease in the magnetization with each respective layer, from 62 to 56 and 31 emu/g. However, the PLL–HP iron oxide nanoparticles still retained useful magnetic properties. The authors visualize that this work can be useful for a delivery system, especially for chemotherapeutic

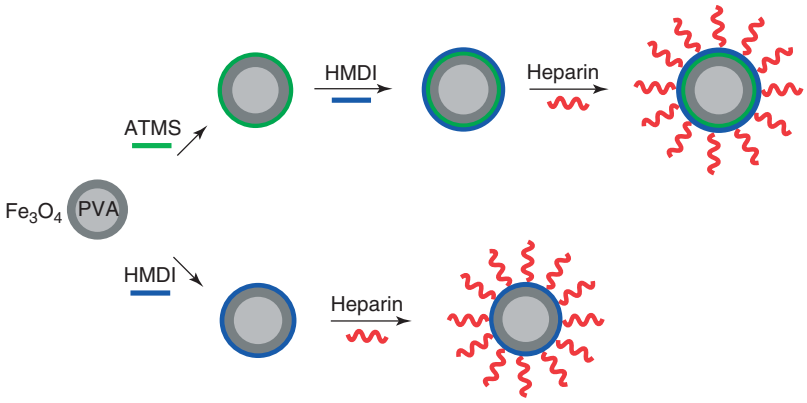

FIGURE 2 | Schematic cutaway of heparinized poly(vinyl alcohol) (PVA)/Fe_3O_4 core/shell nanoparticles. Heparin is conjugated to the outer Fe_3O_4 shell through interaction with the HMDI spacer arm, which is conjugated to either the Fe_3O_4 shell or the aminotrimethoxysilane longer spacer.

cancer treatments. HP can provide biocompatibility to these magnetic nanoparticles, which can be targeted to specific delivery sites and manipulated by magnets to control the metastasis of tumors and perhaps eventually destroy the cancer cells.

Even though HP is predominantly used as an anticoagulant, its ability to interact with many different proteins and display various biological activities has made this a potential candidate for other clinical purposes. One technique used for drug delivery is polymeric amphiphiles, where hydrophilic and hydrophobic components assist in forming micelles that serve as a compartment for various drugs.[42,43] Polysaccharides have been used as the hydrophilic entity of these systems attached to a hydrophobic entity. Specifically, HP is of interest for use as a drug delivery system for treatment of cancers because it has been shown to inhibit angiogenesis and metastasis, particularly nonanticoagulant HP.[44–46] Park et al. developed a self-assembled HP–deoxycholic acid nanoparticle to be used as anticancer drug delivery.[47,48] In this work they use deoxycholic acid (DOCA), a bile acid, as the hydrophobic moiety and HP as the hydrophilic constituent. The carboxyl groups of HP were modified by activation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and coupled to an aminated DOCA, forming amphiphilic molecules. Nanoparticles were then formed by sonicating in an aqueous environment, producing particles ranging from 120 to 200 nm, depending on the amount of DOCA conjugated to HP. Their studies showed a high negative charge to the particles, indicating that HP is forming a shell around the particles, electrostatically stabilizing them in aqueous solution. The authors hypothesized that these negatively charged nanoparticles could interact and inhibit growth factors that are vital to tumor survival.

Park et al. took their work further to investigate the potential of these nanoparticles as drug carriers for cancer therapy.[48] Doxorubicin (DOX), a commonly used chemotherapy drug, was entrapped into the amphiphilic HP–DOCA nanoparticles by hydrophobic interaction with the DOCA moiety of the particles (Figure 3). These particles were tested for their cytotoxicity in mouse models, where no significant or unexpected side effects occurred, while free DOX caused a significant loss of weight in the models. DOX-loaded nanoparticle also significantly inhibited proliferation of squamous cell carcinoma (SCC) and human umbilical vascular endothelial cell, with a ~60% and ~70% inhibition, respectively, compared with free HP. *In vivo* testing on their ability to suppress SCC tumor growth was also evaluated in mouse models showing the entrapped DOX (12%) to the HP–DOCA nanoparticles had the highest suppression of tumor growth compared to free DOX and HP–DOCA nanoparticles. The ability to inhibit growth compared with the free DOX was explained by a prolonged circulation of the DOX-loaded nanoparticles compared with the free DOX, exposing the cancer sites for a longer amount of time with the drug. The authors also explain that these particles can have a dual action of inhibiting cancers, by first destroying the tumor cells with the DOX drug, and second inhibiting cellular proliferation with the HP that is present within the nanoparticles. However, further investigation needs to be done to fully understand the mechanism of inhibition and increase efficiency compared with other DOX-loaded nanoparticle drugs.

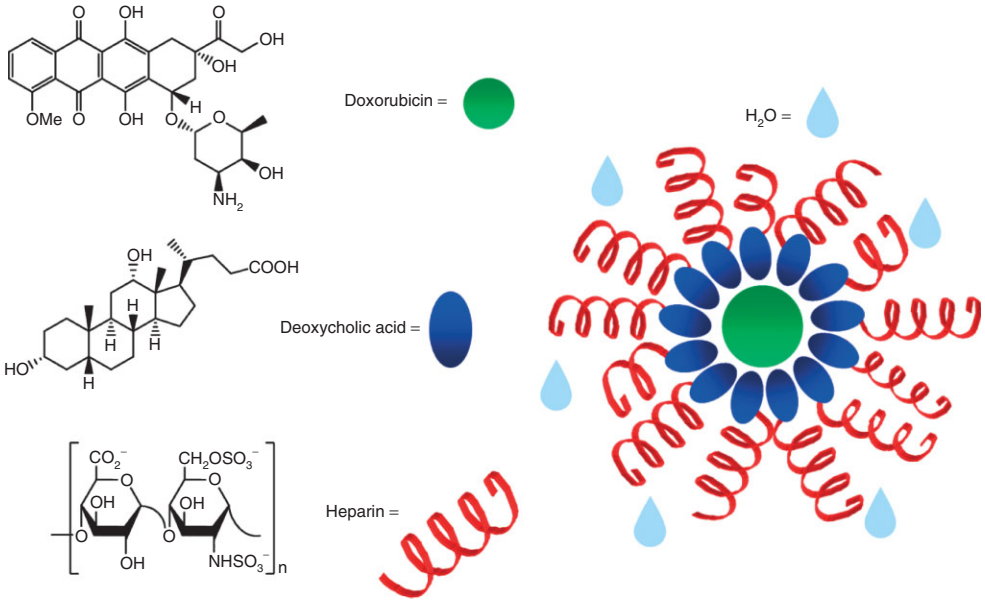

FIGURE 3 | Schematic of self-assembled heparin–deoxycholic acid (HP–DOCA) nanoparticles loaded with doxorubicin (DOX) for cancer treatment. HP and the DOCA compound is an amphiphile that will form micelles that can encapsulate the hydrophobic DOX chemotherapeutic drug.

TISSUE AND BONE ENGINEERING

The uniquely high negative charge of HP allows it to electrostatically interact with positively charged materials, which can form nanoparticles. Na et al.[49] utilized a layer-by-layer fabrication method that relies on strong ionic charges of different polymers and molecules being used. In their initial studies, they were able to fabricate nanoparticles using positively charged PLL and HP. PLGA mircospheres were then coated with a layer of poly(ethyleneimmine) (PEI), which can electrostatically interact with the HP–PLL nanoparticles. The HP–PLL nanoparticles coat ~70% of the microsphere surface (Figure 4). The motivation for this 3D matrix was to make a stem-cell delivery system, which could induce cell growth at an adequate level for engineered tissue regeneration. Conventional methods culture cells as monolayers where scalability is limited. When the mesenchymal stem cells (MSCs) were cultured with the HP–PLL microspheres, the authors saw an increase in cell growth as compared to the monolayer of MSC. A specific marker for collagen type II was used to determine if the MSCs were differentiated to form cartilage. The MSCs that were incubated with just the bare HP–PLL microspheres showed an abundance of GAGs and polysaccharides within the ECM, while the MSCs that were incubated with the PLGA microspheres had limited growth and only produced ECM. Their results confirmed that HP–PLL attached to the PLGA microspheres can enhance cellular adhesion, induce growth, viability, and differentiation of the MSCs for tissue formation. This 3D matrix of HP–PLL nanoparticles conjugated to PLGA microspheres has potential in a variety of biological applications for tissue regeneration, or as cell or protein and peptide delivery systems.

This study was extended to include the immobilization of a transforming growth factor β (TFG-β).[33] Proteins immobilized to solid supports often retain their structural integrity and biological activity over long periods of time because they are less prone to unfolding and aggregation. To direct the MSCs for chondrogenesis for neocartilage formation, specific growth factors are often necessary.[50–52] MSCs can lose their activity and fail to differentiate into cartilage-specific cells, but growth factors can prevent this loss of activity.[53–55] Nanoparticles were analyzed by circular dichroism at 21 days after TFG-β was immobilized. The results showed that the protein still retained its structure and activity to induce GAG, aggrecan, collagen, and COMP (a major marker for hyaline cartilage) production, indicating its ability to cause chondrogenesis of the MSCs for formation of cartilage tissue. This study was an extension of previous work that had fabricated and characterized these 3D matrices. By loading the 3D matrix with a growth factor, MSCs could be directed to differentiate into neocartilage. This system has potential for implantable devices that could improve biocompatibility and promote tissue formation.

Using the same layer-by-layer assembly approach for load-bearing bone applications, Rai et al.[21] used nanohydroxyapatite (n-HA) as the support for layering HP, then poly(L-histidine) (PH), followed by another layer of HP. Nanoparticles are being used as fillers for current bone-engineering scaffolds to reinforce their mechanical properties.[56] However, one problem with nanoparticles is their tendency to aggregate, losing their nanoscale properties. A model system was fabricated to enhance dispersion and stability of the n-HA by immobilizing HP (for

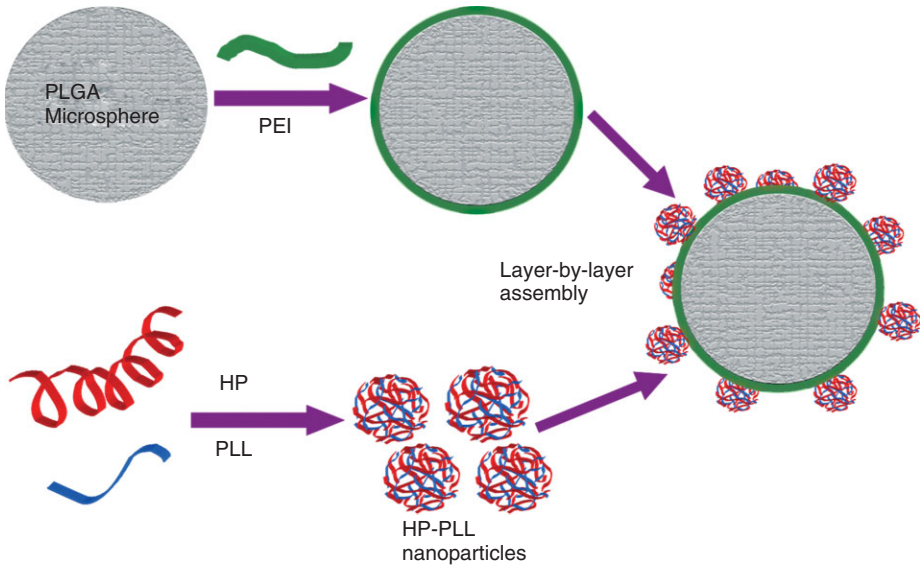

FIGURE 4 | Schematic cutaway of layer-by-layer assembly of poly(lactic-*co*-glycolic acid) microspheres covered with heparin poly(L-lysine) nanoparticles. This assembly relies on the electrostatic interaction of each layer.

stability and localization) and PH (representing growth factors or adhesion proteins). The negative charges of the HP molecules appear to be absorbed onto the n-HA supports. Zeta potential of the composites showed the release of Ca^{2+} after the first layer of HP was immobilized; this indicated that HP was interacting with the n-HA through specific carboxyl and sulfo groups, as opposed to nonspecific electrostatic interactions. The positively charged PH molecules readily absorbed onto the HP layer; followed by another layer of negatively charged HP, the subsequent layers bound to each other more through electrostatic interactions. The charge repulsion after the addition of HP allowed the particles to be stable for a 23-day period of time. This stability is important to enhance the mechanical properties of the nanoparticles within bone-engineered scaffolds. Replacing PH with other positively charged growth factors or adhesion proteins can give these n-HA particles osteoinductive properties, having potential in a variety of orthopedic implants and bone applications.

BLOOD AND BIOCOMPATIBILITY

Nanodevices and nanoparticles for biomedical applications have been well studied for use as delivery systems, implants, and probes. One potential problem in using nanoparticles for *in vivo* applications is their limited blood and biocompatibility. If the nanoparticles or nanodevices are not blood compatible, their injection into the body can elicit a response from the coagulation and complement cascade, where plasma proteins absorb onto the surface, these are then recognized by phagocytes and macrophages, activating the complement cascade, rendering them useless and harmful.[57,58] Passirani et al. used poly(methyl methacrylate) (PMMA) nanoparticles coated with HP or dextran and evaluated their *in vivo* behavior by investigating their ability to inhibit the complement system and blood-circulation time.[17,18] They fabricated PMMA nanoparticles coated with HP chains and found that these particles were able to inhibit the complement cascade.[18] The formation of the nanoparticles is similar to Park et al.[47,48] who used amphiphilic copolymers for self-assembly of the nanoparticles. Passirani et al.[17,18] used PMMA polymer as the hydrophobic component and a carbohydrate as the hydrophilic entity. These copolymers were able to self-assemble into nanoparticles in the absence of surfactant, with an average particle size of $\sim 80 \pm 5$ nm. These particles were determined to have the ability to inhibit complement activation, and work was done to evaluate their half-life within blood. These nanoparticles were formed in a similar way, except they were tagged with a fluorescent probe, *N*-vinyl carbazole, which was used to calculate the amount of particles in the mouse plasma.[17] These particles were slightly larger with a mean diameter of 160 ± 10 nm. These fluorescently tagged HP–PMMA particles and controls were injected into mice, and at different time intervals, blood samples were drawn and the fluorescence was measured. The HP–PMMA nanoparticles had a half-life of 5 h and were still detected in the plasma after 72 h, while the control PMMA nanoparticles had a half-life of 3 min. The ability for these particles to limit the complement cascade and have a prolonged presence in the blood was hypothesized to result from HP forming a 'brush-like' layer onto the surface of the particle, which is able to hide the PMMA surface that can otherwise elicit an immune response, activating the complement cascade, and eliminating the particles from the blood. HP itself inhibits the complement system[12] and can further be used as a 'stealth' molecule for drug carriers, limiting opsonization and phagocytosis by the complement process.

Nanoparticles that are often used for biomedical devices usually have to mimic cells to evade the immune system. HP and other GAGs immobilized to the surface of nanomaterials can mimic the surface of cells that are naturally coated with PGs and GAGs, thus concealing the unnatural nanoparticles from the immune system. Carbon nanotubes (CNTs) are one of the most widely investigated nanomaterial for their incorporation in nanodevices,[59] biosensors,[60] and solid supports.[61,62] Murugesan et al. used CNTs to replace the protein core of HP PGs, forming 'neo-PGs' that can be used for *in vivo* devices.[63] These composites were synthesized by first coating multiwalled CNTs (MWNTs) with PEI through hydrophobic interactions. HP was transformed into its tetrabutylammonium form followed by activation of its free hydroxyl groups with cyanogen bromide, which can then be conjugated to the amine groups on the PEI (Figure 5). This neo-PG was characterized using atomic force microscopy where the author observed a rough surface morphology on the MWNTs with HP attached, compared with pristine MWNTs that appeared to have a smoother morphology. These composites were then tested for their anticoagulant ability using activated partial thromboplastin time (APTT)and thromboelastography (TEG). APTT measures the clotting activity of HP using plasma,[64] while TEG measures the clotting kinetics of whole blood.[65] The APTT of the MWNTs with HP showed a linear dose response for a range of 0.75–3.0 mg/ml; MWNTs–PEI only showed a small anticoagulant effect above a concentration of 1.5 mg/ml, while pristine MWNTs did not show any

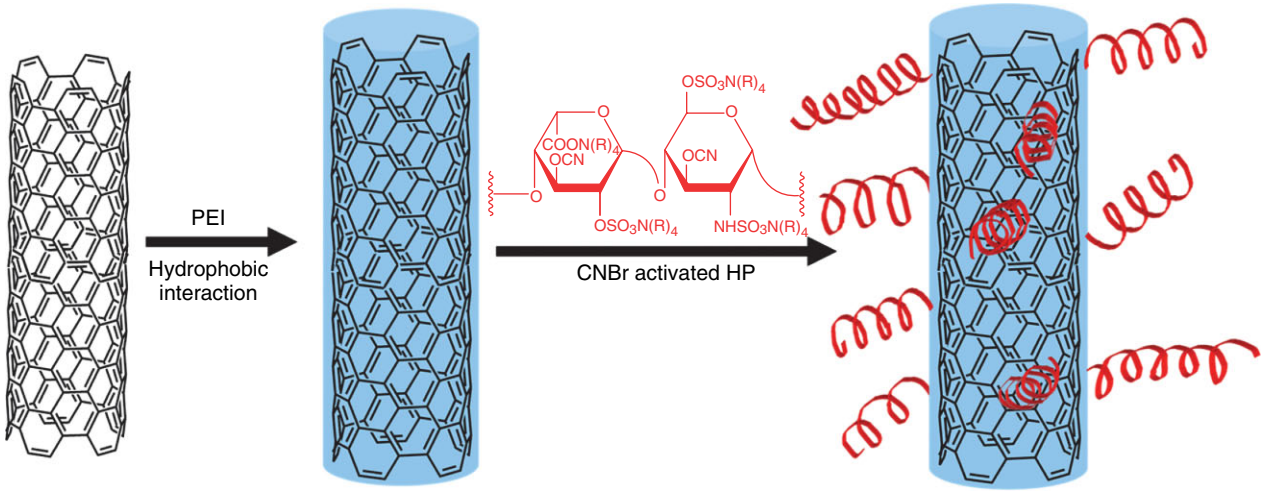

FIGURE 5 | Schematic of neo-proteoglycan. The multiwalled carbon nanotubes are coated with poly(ethyleneimmine), where CNBr-activated heparin can interact with the exposed amine groups.

anticoagulant activity over a range of concentrations. TEG results were similar to the APTT, where heparinized MWNTs had anticoagulant activity. When the heparinized MWNTs were treated with heparinase, which is an enzyme that digests HP, and analyzed by TEG there was a decrease in the ability to inhibit coagulation providing support that HP was immobilized to the surface of the MWNTs. This work provides a way of making blood compatible MWNTs by covalently immobilizing HP, facilitating the use of these composites as building blocks for nanodevices for biological *in vivo* applications.

The conjugation of HP and GAGs to metallic nanoparticles has also been well studied. One approach is the direct synthesis of gold and silver nanoparticles (AuNPs and AgNPs, respectively) with HP.[25,26] The use of polysaccharides and other sugars offers a nontoxic green method for the reduction of metal salts and subsequent synthesis of nanoparticles, avoiding toxic chemical reductants that can be harmful if used for *in vivo* applications. Kemp et al.[27] used this approach to synthesize AuNPs and AgNPs using HP and hyaluronan, another GAG. The synthesis of these particles was a simple, one-pot reaction that uses the reducing end of the HP chains to reduce $AuCl_4$ and $AgNO_3$. HP was derivatized with a 2, 6-diaminopyridine group (DAP) at the reducing end; the amine groups on the DAP can interact with the surface of the AuNPs or AgNPs. The interaction between the DAPHP and the nanoparticles provide stability under physiological conditions and can control the size of the particle being formed. The average size distribution for AuNPs and AgNPs reduced with DAPHP was 10 ± 3 nm and 7 ± 3 nm, respectively (Figure 6). The HP chains were digested from the nanoparticles and quantified using carbazole assay,

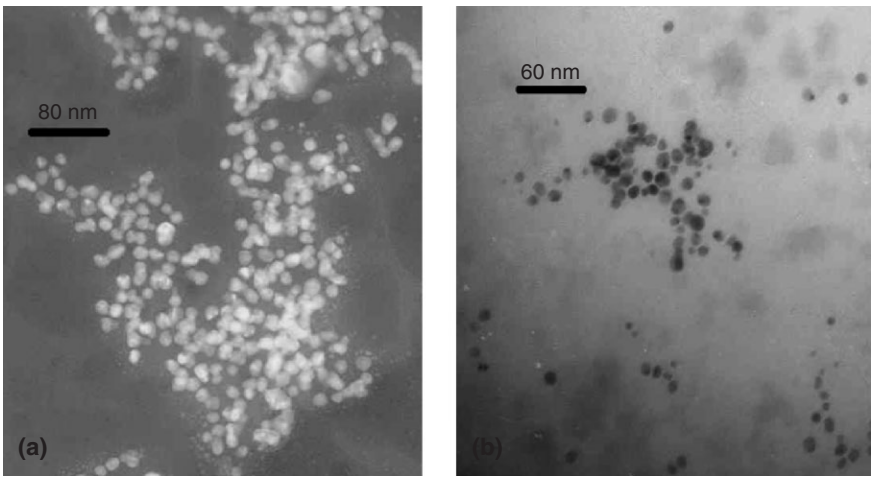

FIGURE 6 | Transmission electron microscopy images of 2,6-diaminopyridine heparin (a) gold nanoparticles at 125,000× magnification and (b) silver nanoparticles at 160,000× magnification.

which detects the uronic acids of HP. Based on the amount of HP and theoretical calculations of the AuNPs and AgNPs formed, it was determined that ~50 HP chains and ~20 HP chains were attached to one AuNP and AgNP, respectively. The HP particles were tested for their anticoagulant ability using APTT; the HP nanoparticles showed activity similar to the free DAPHP to inhibit clot formation. These particles were also tested for their anti-inflammatory activity, as gold salts have been previously used to treat arthritis. The HP nanoparticles showed a slight reduction in inflammation, but not as potent as the control drug, indomethacin. After the animals were euthanized, their blood was taken and analyzed by APTT. Free HP appeared to diffuse through the body and was present within the blood, preventing coagulation. While the blood of the rat treated with HP nanoparticles did not show anticoagulant activity, it appears that the nanoparticles provided localization of the drug to the site of injection. This localization is useful to provide prolonged effects and prevent systemic reactions of injecting the drug that can otherwise diffuse throughout body. These particles coated with HP can increase stability, localization, and biocompatibility for use in *in vivo* applications.

CONCLUSION

HP is a widely studied GAG, having many biological roles, the most important being its anticoagulant activity. The ability for HP to inhibit the coagulation and complement cascades makes it an ideal candidate for passivating and imparting blood compatibility to surfaces. The combination of nanoparticles with HP has emerged as an intensely studied field. Nanoparticles incorporated with HP molecules show increased intrinsic anticoagulant, anticomplement, and other biological activities. HP chains also mimic cell surfaces on the nanoparticles to evade the body's defenses and immunological system. This myriad of applications makes these composites a particularly rich area for future study.

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