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HEPARIN DERIVED NANOCARRIERS AND HYDROGELS IN BIOLOGICAL DELIVERY

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ABSTRACT

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Heparin is a glycosaminoglycan, which is known as an anticoagulant. Non-anticoagulant properties of heparin, such as its antiangiogenic and antitumor activities, are studied extensively at the moment. Heparins can be modified either by structural modifications, or by conjugating other components into it, like metals, polymers, or proteins. There is a large variety or different heparin derivatives with many applications, including nanocarriers and hydrogels. Heparin-based nanocarriers allow safe and targeted delivery for many drugs, meanwhile heparin-based hydrogels participate in controlled and sustained release of growth factors for tissue regeneration. A class of hydrogels, nanogels, have similar role to nanocarriers as they are able to deliver drugs and biomolecules in a targeted manner. The future prospects for heparin derived nanocarriers, hydrogels and biomaterials are promising, and many wound dressings and medicine based on them are already in clinical testing.

TIIVISTELMÄ

Hepariini on glykosaminoglykaani, joka on tunnettu antikoagulantti. Hepariinin ei-antikoagulanttisia ominaisuuksia, kuten antiangiogeenisiä ja kasvaimen vastaisia ominaisuuksia, tutkitaan paljon tällä hetkellä. Hepariineja voidaan muokata joko rakenteellisesti, tai konjugoimalla siihen muita aineita, kuten metalleja, polymeerejä tai proteiineja. Erilaisten hepariinijohdannaisten määrä on suuri, ja niillä on sovelluksia mm. nanokantajina ja hydrogeeleinä. Hepariiniin pohjautuvat nanokantajat mahdollistavat monien lääkeaineiden kohdennetun ja turvallisen kuljetuksen. Hepariiniin pohjautuvat hydrogeelit ovat osana kasvutekijöiden ohjattua vapauttamista kudosvaurioiden parantamiseksi. Hydrogeeleihin lukeutuvat hepariinista johdetut nanogeelit toimivat nanokantajien tavoin, eli ne voivat kuljettaa lääkkeitä tai biomolekyylejä kohdennetusti. Hepariinista johdettujen nanokantajien, hydrogeelien ja biomateriaalien tulevaisuudennäkymät ovat lupaavat ja monia niihin perustuvia lääkkeitä ja haavasiteitä on kliinisissä testeissä.

Key words: heparin, nanocarrier, hydrogel, biomaterial, drug delivery, growth factor delivery, cancer therapy, heparin derivative

PREFACE

This is a literature review about heparin-derived nanocarriers and hydrogels in biological delivery, and it serves as my Bachelor's thesis.

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SISÄLLYSLUETTELO

1. INTRODUCTION

Heparin is a linear glycosaminoglycan, which consists of 1-4 linked disaccharide units made of uronic acid and glucosamine (Liang and Kiick 2014). In nature, it is mainly found in mast cells, from where it is released alongside histamine. Heparin has a role at preventing inflammation from spreading since it binds to many proteins present in the inflammation process, such as cytokines, adhesion molecules and growth factors. Therefore, heparin could potentially be used to treat some inflammation diseases in human (Lever and Page 2002).

Heparin is also a well-known anticoagulant, and it has been used as blood anticoagulant since 1930s (Yang et al. 2015). In blood circulation it binds to antithrombin, which in turn inactivates thrombin that has an important role as coagulant, thus stopping the coagulant cascade. In clinical use chains without wanted biological effects or possibly with harmful effects such as anticoagulant activity, can be removed from heparin (Lever and Page 2002).

Heparin has many antitumor activities. Its antimetastatic activity can prevent the adhesion and extravasation of metastatic cancer cells by interacting with some of the involved molecules. For example, heparin can decrease the activity of heparanase, which destroys the extracellular matrix (ECM) and is overexpressed in cancer or compete binding with P- and L-selectins, which mediate cell-cell interactions necessary in tumor metastasis. It is also antiangiogenic, so it prevents blood vessels forming in the tumor by binding to angiogenic growth factors necessary for neovascularization. Heparin is able to interact with transcription factors as well and can cause apoptosis to the target cell (Liang and Kiick 2014; She et al. 2013; Yang et al. 2015). The targeting ability of heparin is based on interacting with the network of coagulated plasma proteins found on the vessel walls and stroma of the cancer. Heparin has also a targeting activity against rapidly dividing endothelial cells found in the cancer and heparin-binding angiogenic growth factors, which are over-expressed in the cancer (Yang et al. 2015).

Many different biomolecules can bind to heparin due to its abundance of functional groups. Heparin promotes cell adhesion, enables cell-mediated proteolytic degradation, improves biocompatibility and therapeutic effect of a biomolecule as well as allows the controlled delivery of growth factors (Liang and Kiick 2014). Chemically modified heparin derivatives have less anticoagulant activity and more efficient antiangiogenic effects. There are many different heparin derivatives, but the derivation of the carboxyl groups of heparin is often used (Yang et al. 2015).

Heparin has various unique biological and physiochemical qualities as a result of its structure and interactions with biomolecules, such as antitumor activity, non-toxicity, non-immunogenicity, biodegradability, solubility, ability to carry proteins and targeting ability. It can also stabilize growth

factors and enhance their affinity to their receptors, and it has a good affinity to many other biomolecules as well (Yang et al. 2015). These qualities make the use of heparin compelling in nanocarriers, hydrogels and surfaces of biomaterials.

Nanocarriers have many clinical applications including cancer medicine, gene therapy and diagnostics. They have been made to reduce disadvantages of free therapeutics and conventional delivery, such as poor distribution, stability, or solubility. They also increase the safety and efficacy of their cargos and prolong their circulation times. Nanoparticles can be lipid-based, inorganic, or polymeric (Mitchell et al. 2021). Incorporating biological molecules on or within the nanoparticles enhances their activities and biocompatibility (Kemp and Linhardt 2010).

Hydrogels are polymeric materials with hydrophilic groups. They are insoluble in water but can swell due to their crosslinked three-dimensional structure. They can be classified in various ways, such as based on their size, origin, application, and network binding approach. Their general advantages are hydrophilicity, biocompatibility, non-toxicity, and degradability (He et al. 2019). They have various applications that depend on their properties and structure. Soft hydrogels with an ability to respond to stimuli and exchange material could be used in biosensors, artificial organs, and wound dressings. Biocompatible hydrogels with higher molecular masses, higher charge density and muco-adhesive properties can be used in controlled drug delivery (Wenbo et al. 2020).

2. HEPARIN DERIVATIVES

Heparin can be divided into two groups. Unfractionated heparin (UFH) is a sulfated glycosaminoglycan with very heterogeneous chemical structure and molecular weight. It has many many sulfate and carboxylate groups, which give it a strong, negative charge, which in turn mediates electrical interactions with its target proteins and make it highly hydrophobic (Yang et al. 2015). Because of its anticoagulant activity, the side effects of UFH include hemorrhagic complications and thrombocytopenia, which causes the immune system to target heparin and create antibodies against it, forming blood clots (Hwang and Lee 2016). There are also issues with the supply and safety since it is often extracted from natural tissues in animals, such as porcine intestinal mucosa or lung and intestine from cattle (Liang and Kiick 2014; Yang et al. 2015). The animal origin and biosynthesis often cause highly variant and heterogenous chain lengths and sulfation patterns. Advantages of UFH include fast effect, low price, no placenta passage, and neutralization in body by protamine as its antagonist (Laner-Plamberger et al. 2021).

Low-molecular-weight heparin (LMWH) is derived from UFH by chemical and enzymatic depolymerization, and it compensates with the side effects and issues of UFH, since its chemical structure is defined better. The biological activities and properties of LMWH are at least as good than those of UFH (Fernández, Hattan, and Kerns 2006; Yang et al. 2015). LMWHs show better subcutaneous bioavailability and longer half-life than UFH, since they have lower affinity for plasma proteins and blood cells. They also cause fewer adverse reactions, so LMWHs have almost completely replaced UFH in clinical practice (Laner-Plamberger et al. 2021). LMWHs are still structurally inconsistent, since they are produced by different depolymerization methods for different applications, meaning they have different properties to each other (Ji et al. 2020). There are also so-called synthetic ultra (U)LMWHs, which consist only of five to ten saccharide molecules, and are used as efficient anticoagulants by being selective inhibitors to activated blood coagulation factor X (Laner-Plamberger et al. 2021).

In nature, negatively charged heparin binds to cationic regions in various proteins, which are called heparin-binding proteins. Heparin can be chemically or biosynthetically modified to control its binding to specific proteins or to add or remove features to it. The most common modifications to heparin structure are altering sulfate substitution patterns, carboxyl reduction, chain fragmentation and replacing N-sulfo groups with N-acetyl groups. By incorporating synthetic moieties, such as aliphatic and aryl moieties, to heparin, semi-synthetic heparin derivatives are created. One way to create heparin that selectively binds to some proteins over others is to replace N-sulfo groups on heparin with non-anionic moieties to create reduced-charge heparin derivatives (Fernández et al. 2006).

The antithrombin binding sequences of heparin determine its anticoagulant activity. At first, heparin derivatives focused on keeping heparin as an anticoagulant but later the heparin structure has been modified more strongly and the anticoagulant activity has been reduced (Fernández et al. 2006). This antithrombin binding region (ATBR) needs to be inactivated in order to reduce the risks of bleeding to use heparin for non-anticoagulant purposes (Alekseeva et al. 2017). The anticoagulant activity of heparin can be decreased with carboxylate, amino and alcohol modification as this disrupts its interaction with thrombin (Hachim et al. 2019). To do this, enzymatic digestion and affinity chromatography are the most selective methods, but due to their cost and time-consuming nature, glycol-splitting of uronic acid residues by periodate oxidation is another way to inactivate ATBR without affecting other biological activities of heparin (Alekseeva et al. 2017). It is also observed that the molecular weight of heparin in itself is a component in its activity against thrombin, meaning that shorter heparin does not usually inactivate thrombin enough, thus it has less anticoagulant activity (Hwang and Lee 2016). There is preclinical evidence that the antitumor properties of heparin are not connected to its anticoagulant and antithrombotic activities (Lanzi and Cassinelli 2018).

Typical chemical modifications of heparin include changing chain-length, chain integrity, and substitution patterns of anionic groups. Other type of chemical modification is modifying glucosamine C-2-amino groups in heparin, which includes both amine substitution after N-sulfonation, and direct N-acylation of heparin using its natural amino groups. Former modification is reported to have both anti-HIV and anti-metastasis activities and latter modification allows the use of heparin derivative as chemical probes including fluorescent tags (Fernández et al. 2006). N-acetylated, N-resulfated and N-desulfated heparins are shown to have heparanase inhibitory activity as their antimetastatic activity has been observed, and additionally they keep their anti-inflammatory properties even with reduced anticoagulant activity (Lanzi and Cassinelli 2018). Derivatizing the carboxylate groups of heparin creates either heparin esters or heparin amides via carboxylate substitution, meanwhile it is also possible to derivatize heparin through O-acylation and O-alkylation. Lastly, heparin derivatives can be attained by chemical modification at the reducing end, which can be used, for example, to attach chemical or fluorescent probes into heparin (Fernández et al. 2006).

Modifying heparin can make it hydrophobic or even add functional and crosslinking moieties useful for scaffold construction. Substituting the carboxylic acids on the uronic acid residues is the most common heparin modification but modifying amino groups on their non-acetylated or non-sulfated sugar residues is also typical. Physically constructed heparin can have diverse mechanical properties, but it can also be just a minor factor in a scaffold that gives the wanted mechanical or structural properties. The different forms of heparin can be used in hydrogels, surface coatings, nanoparticles, coacervates, and scaffolds. Adding new properties with chemical modification enables for example an addition of methacrylate groups to heparin for crosslinking with PEG-hydrogel (Hachim et al. 2019).

Alyahya et al. compared the anti-metastasis effectiveness and safety of heparin derivative with no anticoagulant properties and LMWH in surgical pancreatic cancer models. The antimetastatic properties of heparin are caused by inhibition of heparinase, blocking of both P- and L-selectins, and inhibition of angiogenesis. LMWH is known to reduce tumor metastasis both in animal experiments and in clinical trials, but it has caused more bleeding in treated patients. Sulfated non-anticoagulant heparin (NACH) derivatives were created to decrease the anticoagulant activity of heparin. The efficacy of two heparins was studied with IVIS imaging and histopathological studies showed that NACH had better anti-tumor properties and had shorter bleeding time than LMWH. The study showed that heparin derivative with reduced anti-coagulant activity was more effective and safe compared to LMWH, which was an antithrombotic drug (Alyahya et al. 2015).

Commercially available LMWHs with high anticoagulant activity are produced by chemical or enzymatic degradation of unfractured heparin. They are produced by different depolymerization methods, which causes a great amount of structural inconsistency and variation between different

LMWHs. As a result, studies of the non-anticoagulant activities of LMWHs have inconsistent outcomes. For example, in one study comparing two different LMWHs it was found out that they had completely opposite effects on cytokine release. Glycoengineering techniques are required to expand and control the heparin-derivative structures with decreased anticoagulant activity. Ji et al. produced a library of heparin derivatives with diverse structures and decreased anticoagulant activity by combining enzymatic cleavage and chemical modifications of heparin. Heparinases I-III with different substrate specificities and as different combinations were used to create diverse heparin sequences, and by switching the order of chemical modification and heparinase polymerization, even more diversity was created. Multiple heparin derivatives were also screened in cell apoptosis model, and it was observed that partially 6-O/N-desulfated LMWHs demonstrated the most potent anti-apoptotic activity, so it was clear that anti-apoptotic activity is not dependent on the anticoagulant structure of heparin (Ji et al. 2020). Anti-apoptotic activity of heparin is based on upregulating genes affecting apoptotic cell death as a result of its interactions with transcription factors (Liang and Kiick 2014).

Meanwhile heparinases can be used to cleavage heparin, heparanase is an endoglycosidase, which degrades heparan sulfate (HS) chains in the ECM. It is often overexpressed in cancer as it promotes tumor metastasis and angiogenesis, but heparin can act as its inhibitor due to its similar structure to HS. Periodate oxidation and borohydride reduction of the non-sulfated uronic acid residues of heparin inactivates the ATBR and creates glycol-split (gs) heparins. Non-anticoagulant gs heparins produced by this method, such as an anti-cancer drug Roneparstat, show high heparanase-inhibitory activity compared to UFH as well as high anti-tumor and anti-metastasis activities (Alekseeva et al. 2017). Gs heparin chains are more flexible than normal heparin, and they are antagonistic for the construction of the ternary complexes with growth factors and their receptors (Lanzi and Cassinelli 2018). As a result of their modification, gs heparins are poor substrates for heparanase, which keeps them away from degrading and losing their activity. In addition, their lower sulfation degree reduces unspecific interactions with other proteins, so they become very selective in inhibiting heparanase or some other proteins, like growth factors (Alekseeva et al. 2017).

Preclinical oncology studies have shown heparin derivatives to potentially prevent every phase of cancer development and progression, including growth of the tumor, angiogenesis, and metastasis. Supersulfated LMWH is created via depolymerization with oversulfation. The heparin derivative interferes with the activities of multiple pharmacological targets, such as heparanase and growth factors, with reduced anticoagulant activity. It has shown clear antitumor and antimetastatic activities both in vitro and in vivo as it has inhibited growth and invasion of cancer and downregulated the activation of various growth factor receptors. Supersulfated heparins have also demonstrated

hepcidin inhibition, which regulates iron metabolism involved in cancer as well. Sulfated low-anticoagulant heparin has also shown antimetastatic activity by inhibiting P-selectin in mouse pancreatic cancer model. The 2,3-O-desulfated heparin CX-01, which is in clinical evaluation to be used as an adjuvant to chemotherapy, and many other heparin derivatives only show significant antitumor activity when used in combination treatments. However, since heparin derivatives are known for their good safety and tolerability profiles, they could be used to enhance the antitumor efficacy of many conventional treatments (Lanzi and Cassinelli 2018).

The capacity to regulate the activity of heparin by chemical and physical conjugation while keeping its angiogenic properties has been studied. The anti-cancer properties of heparin are improved by forming heparin derivatives, where heparin is conjugated to bile acids, polymers, proteins, or metal nanoparticles to enhance its availability for drugs and anti-angiogenic properties, and to reduce the anticoagulant activity of heparin. Bile acid-conjugated heparin provides lower anticoagulant activity and hydrophobicity due to modified structure. When amphiphilic deoxycholic acid is chemically conjugated to heparin by forming an amide bond, it induces the oral absorption of heparin in the intestine using the bile acid receptor, so it is possible to use it as an orally absorbable anti-cancer medication (Hwang and Lee 2016). Oral administration of heparin derivative conjugated to deoxycholic acid has shown remarkable antiangiogenic and antitumor activity in carcinoma models (Lanzi and Cassinelli 2018). Chemically conjugating taurocholic acid to heparin has significant anti-angiogenic properties with less anticoagulant activity compared to unmodified heparin in tumor xenograft model. Conjugating heparin to antiangiogenic proteins, like endostatin, can have combined effect on inhibition of cancer progression, and heparin-endostatin has indeed shown high activity in inhibiting the process of angiogenesis. On the other hand, some metal nanoparticles, like silver and gold, are biocompatible with studied anticoagulant activity and anti-inflammatory effects, so synthetizing them to heparin can enhance these effects synergistically (Hwang and Lee 2016).

3. HEPARIN-BASED NANOCARRIERS

3.1 Structure and properties of heparin-based nanocarriers

Carrying and delivering bioactive agents are some of the many advantages of nanocarriers. They can deliver agents, such as drugs or biomolecules, either encapsulated inside their core or absorbed onto their surface (Yang et al. 2015). The half-life of many biomolecules, like proteins, is short in the extracellular environments so without nanoparticle systems to carry and stabilize them, they would degrade before reaching the target tissue (Dai Hai Nguyen et al. 2011). Nanocarriers

can ensure the safety and the efficacy of cytotoxic drugs by taking advantage of both active and passive targeting strategies (Yang et al. 2015). Enhanced permeability and retention effect (EPR-effect) causes the accumulation of nanocarriers and thus the drug to the tumor site (She et al. 2013). Additionally, they can deliver greater amount of drug to the tumor sites using the tumor targeting ability of heparin, meanwhile the amount of drug is minimal in other tissues compared to non-specific distribution of free drug, which can cause harmful side effects. There is also a controlled way to make nanocarriers release their load by using the environment, for example utilizing biodegradability, pH, ion, or temperature sensitivity of the nanocarrier material. It is possible to load multiple different substances inside the nanocarrier, which can be used in combinatorial therapy (Yang et al. 2015).

Nanocarriers first travel in the circulation, where they interact with blood proteins and fluid forces. They reach the target site via extravasation from capillary walls, which have larger intercellular gaps in tumors. The properties of the nanocarrier determine, whether it reaches the target tissue or not, so they need to be well defined. At the target site, nanocarriers cross the local microenvironment with often different conditions compared to the circulation, including lower pH in many tumors and hyperthermic wound sites. It is possible to create nanoparticle systems, which release their cargo in specific pH or temperature to create targeted delivery (Mitchell et al. 2021).

Before administration, the physiochemical properties, including size, shape, charge, and surface coating, of the nanocarrier should be fully defined (Choi et al. 2021). The diameter of nanoparticles should be between 10 nm to 100 nm so they can take advantage of the EPR-effect. If the diameter is too small, the nanocarrier often cannot avoid renal clearance or it can leak into blood capillaries and when the diameter is too big, it is easily captured by macrophages from the reticuloendothelial system (RES) or it can activate the complement system (Ai et al. 2018; Mitchell et al. 2021; She et al. 2013). The surface charge of nanoparticles is important as well for decreasing the non-specific uptake and swift clearance. Positively charged nanoparticles are quickly cleared out from the plasma meanwhile neutral and negatively charged nanoparticles, including heparin-based nanoparticles, decrease plasma protein adsorption and non-specific cellular uptake (Choi et al. 2021).

Heparins, like many other glycosaminoglycans, are biomaterials with useful and wanted properties for biological delivery. Heparin can sequester great amount of proteins and release them in a controlled and sustained manner. It can also enhance or regulate the biological effects of delivered proteins or act as a co-factor to create a synergetic effect. The specific interactions between heparin and its target proteins are often electrostatic interactions between the anionic carboxylate or sulfate group of the heparin and basic amino acids of the target proteins, but they can also be formed by hydrogen bonding, Van der Waals forces or hydrophobic interactions (Hachim et al. 2019; Lanzi and Cassinelli 2018).

The main advantages of heparin nanocarriers compared to nanocarriers made of synthetic materials are its non-immunogenic, non-toxic, and well-defined properties (Ai et al. 2018). It is also able to sequester and interact with proteins it delivers, like growth factors and cytokines, or act as co-factors to regulate binding to their receptors (Hachim et al. 2019). For example, liposomal nanocarriers are unstable and poorly soluble, meanwhile polyethylene glycol (PEG) is not biodegradable and can cause an immune reaction and additionally carbon nanotubes are not very biocompatible (Ai et al. 2018). As natural biomaterial, heparin has little immunogenicity in comparison to synthetic biomaterials and has even showed to inhibit the recruitment and adhesion of leukocytes when applied as coatings for implants. While heparin is the most used glycosaminoglycan for biological delivery, it is known to have off-target binding, which can cause negative side effects or even prevention of processes needed for tissue homeostasis and regeneration (Hachim et al. 2019).

Heparin and its derivatives allow therapeutics to be encapsulated within the nanoparticle core, entrapped in the matrix of the polymer, conjugated to the polymer chemically, or bound to the surface of the nanoparticle (Mitchell et al. 2021). When heparin is used as the backbone of nanocarrier, heparin chains have the main role in forming of the particles, which often happens as a self-assembly of amphiphilic heparin derivatives. This type of nanocarrier usually has a hydrophilic heparin coat and hydrophobic inner core, where the insoluble agent is loaded. The loaded agent is attached either by physical encapsulation or by chemical conjugation. With heparin-drug conjugate nanoparticles, hydrophobic drugs are either linked chemically straight to heparin chains or they are added inside after the polymeric conjugates are formed, in which case they are encapsulated in the core during the self-assembly of the particle. A common approach for these nanoparticles is heparin scaffold working as the carrier, drugs conjugated to heparin and a biological response linker. This nanoparticle enhances solubility of hydrophobic drugs, prolongs their half-time in plasma, protects them from degradation, changes their distribution due to EPR-effect and allows their triggered release with right stimuli. Heparin-based polymeric conjugate nanoparticles are synthetized differently by adding hydrophobic chains to heparin, which aids their self-assembly in solution. The hydrophilic coats of these nanocarriers stabilize them and lengthen their circulation time in blood. It is necessary to choose nonimmunogenic and nontoxic material with drug-loading ability for the hydrophobic core as it interacts with the insoluble drugs. Furthermore, it is possible to conjugate additional targeting molecules to heparin backbone, which makes the delivery more specific. Polyelectrolyte complex nanoparticles (PCN) are also included with nanocarriers with heparin as backbone. They are based on electrostatic interaction, utilizing the high electronegativity of heparin (Yang et al. 2015).

Nanoparticles coated with heparin and its derivatives enhance the stability of the nanocarrier and prevent them from being eliminated by RES cells. They can also target tumor sites with heparin as a functional moiety due to the tumor-targeting ability of heparin. Additionally, they enhance the drug

loading, exploit the various biological activities of heparin, and increase the cellular uptake of nanoparticles (Yang et al. 2015). There are multiple common approaches in functionalizing nanoparticle surface, such as covalent coupling, noncovalent attachment, and physical encapsulation (Liang and Kiick 2014). The modified core of the nanocarriers can be made from very different materials depending on the wanted properties and application. Heparin-coated inorganic nanoparticles include magnetic nanoparticles, for instance iron oxide, and gold nanoparticles. Nanoparticles with magnetic core can be controlled with magnets even after the modification, increasing their targeting ability and even making it possible to control the metastasis of tumors. Conjugating heparin to magnetic nanoparticles also improves their biocompatibility and they have been used for recycling anticoagulants (Kemp and Linhardt 2010; Yang et al. 2015). On the other hand, gold nanoparticles are more biocompatible than iron oxide (Yang et al. 2015). Using heparin with inorganic materials prevents their colloidal aggregation, stabilizes them, makes them soluble, as well as increases both targeting ability and cellular uptake (Liang and Kiick 2014). When modifying organic nanoparticles, such as liposomes, with heparin, it prevents interactions between the nanoparticle and the blood proteins as well as avoids phagocytosis. Inorganic nanoparticles with heparin chains are also able to inhibit the complement cascade and phagocytosis. Since the surface of cells is typically covered by glycosaminoglycans, the heparin can be used as a stealth molecule for drug carriers. (Kemp and Linhardt 2010; Yang et al. 2015).

3.2 Heparin-based nanocarriers for cancer therapy

Nanoparticles are commonly used in cancer therapy to deliver cytotoxic antitumor drugs. Heparin-nanocarriers are promising for this task due to their natural antitumor activities, including antiangiogenic and antimetastatic properties. They can enhance the effect of the therapeutics by inhibiting tumor growth and metastasis by interacting with biomolecules expressed in tumors. She et al. used heparin-drug conjugate nanoparticles as pH-responsive drug delivery vehicles for cancer therapy. They connected dendrimers with heparin as the core to create self-assembling and soluble dendronized heparin with doxorubicin (DOX) drug covalently linked to the polymer. The structure of the nanoparticle is typical with hydrophobic core containing the DOX-molecules and hydrophilic heparin shell. It was observed that the nanoparticles released their contents faster in pH 5,0, which is the pH corresponding to endosomes and lysosomes, than in pH 7,4 in blood. In the study, mice with tumors were injected with dendronized heparin nanoparticles to assess the antitumor activity and toxicity. The result was that the treated mice showed significant growth retardation and less microvessel density compared to the control group and other groups. Meanwhile free drug DOX is cytotoxic, nanoparticles allowed its slow elution into the tumor and high accumulation. The properties of heparin and dendrimer as a carrier prevented multifocal metastasis, improved antitumor

efficacy, and had no observed toxicity, which is partly caused by their biodegradability (She et al. 2013).

Targeting moieties, such as antibodies or integrin ligands, are added to nanocarriers to prevent non-specific distribution. The moieties interact with the molecules on the surface of the target cell, which are often receptors or ligands overexpressed in tumors (Mitchell et al. 2021). Heparin can act as a targeting moiety because it has a high binding affinity to angiogenic growth factors, which are overly expressed in the tumor tissues (Liang and Kiick 2014). There are also many other biomolecules overexpressed on the tumor cell membrane, which could be targeted for tumor-targeted drug delivery.

It is common for antitumor drugs to be severely toxic with nonspecific distribution in tissues and serious adverse effects. This is true for cis-diamminedichloroplatinum (DDP), but Ai et al. overcame it using an integrin targeted drug delivery system iRGD-heparin nanocarrier (iHP). It is made with succinic anhydride-modified heparin as the frame and conjugating it with iRGD-peptide, which can interact with both integrin and neuropilin-1 receptors and penetrates tumors efficiently. Modifying tumor targeting drug delivery systems with targeting ligands like iRGD that bind to tumor-associated markers, it's possible to raise concentration and penetration of drugs at the tumor sites selectively. The cell internalization happens via complex binding reaction between RGD-motif of the iRGD-peptide and αv-integrins on tumor tissue. Thanks to its structure and presence of heparin, the nanocarrier is very biodegradable and biocompatible without anticoagulant activity and has spesific targeting to integrin, which is overexpressed in tumor tissues. It was observed that iHP-DDP nanoparticles showed significant antitumor activity without weight loss or damage in tumor-bearing mice. The properties of conjugated iRGD-peptide improved the selectivity and tumor penetration ability of the heparin nanocarrier but even the untargeted heparin-DDP nanocarriers accumulated in the tumor tissue via EPR-effect. IRGD-heparin nanocarriers also enhanced the delivery of imaging agents into the tumor. The study demonstrated that adding targeting moieties to heparin increases its distribution to the tumor site (Ai et al. 2018).

Unlike many cancer therapeutics, tumor necrosis factor-related apoptosis inducing ligand (TRAIL) has selective cytotoxicity to cancer cells, so it is not harmful to normal cells. It binds to death receptors that are overexpressed on the surface of cancer cells and induces cell apoptosis. However, its physiochemical instability and short half-life limit any further clinical applications. Its poor pharmacokinetic properties can be overcome by molecular modification and attaching it to PEG. These modifications still lack targeting ability and have poor drug distribution, so Choi et al. encapsulated TRAIL into nanocarriers with LMWH-taurocholate conjugate (LHT7) as an inhibitor for angiogenesis. This nanocomplex took advantage of the antiangiogenic nature of heparin meanwhile the anticoagulant activity of heparin had been lowered. Histological analysis of tumor xenograft model

showed that the treatment with LHT7 had clear pro-apoptotic and anti-angiogenic effects and additionally the tumor distribution at the tumor site was 4 times greater than with free TRAIL (Choi et al. 2021).

A completely different method for cancer-treatment is targeted systematic gene delivery. Localized transfer of genetic materials to the target cells has been researched but limiting off-target gene expression has proved to be difficult. Nanocarriers are often cleared from circulation by lungs and liver, which can create toxicity or unwanted side effects if they accumulate in there. Gene carriers sensitive to external localized physical triggers improve target selectivity. Ultrasound-targeted microbubble destruction (UTMD) is based on the collapsing of gas-filled microparticles called microbubbles under ultrasound directed to the target. While they enhance gene transfer at the target sites due to related mechanical effects that enhance the tissue and cell membrane permeability, they still are also distributed passively and can cause off-target gene expression. PEGylation of the nanocarrier surface reduces nonspecific gene transfer but inhibiting passive gene transfer in general proved to enhance selectivity. Since cationic nanoparticles mediate cellular transfection through electrostatic interactions with anionic proteoglycans on the cell surface, engrafting heparin onto the surface of these nanoparticles could block and inhibit the transfection in both the target and the non-target tissues. UTMD-based gene transfer is separated of the molecular interactions on cell surface, so it cancels the heparin-based block of gene transfer and enables target-selective gene delivery with the use of ultrasound. Chertok et al. created an approach to spatially control gene expression with nanocarriers using heparin masking and UTMD. As a result, engrafting heparin onto the surface of the cationic nanoparticles decreased off-target gene expression in the liver over 700-fold compared to controls while the gene transfer to tumor-site was also enhanced over 10-fold. The study proves that heparinization of DNA-nanocarriers in conjugation with localized activation of gene transfer by UTMD is a promising method to control gene expression spatially (Chertok, Langer, and Anderson 2016).

4. HEPARIN-BASED HYDROGELS

4.1 Properties and structure of heparin-based hydrogels

Most frequently used heparin-based biomaterials include heparin-functionalized surfaces, heparin-based hydrogels, and earlier mentioned heparin-containing nanoparticles. Heparin enhances biocompatibility and efficiency of the hydrogel. When conjugating biocompatible materials with heparin, they provide a proteoglycan-like structure, which mimics the physiological functions of heparan

sulfate, and promotes cell proliferation and differentiation (Laner-Plamberger et al. 2021). The abundance of functional groups in heparin makes conjugation to other biomolecules possible and as follows they advance cell adhesion, cell-mediated degradation of proteins and they can control the loading and releasing of the molecules (He et al. 2019). When the polymer structure in hydrogel contains various functional groups, like when using heparin, it provides stable physical properties, and a possibility for carrying and releasing small molecules (Wenbo et al. 2020). Common applications of heparin-based hydrogels are implantations, tissue engineering, biosensors, and biological delivery (He et al. 2019). They are also used as antithrombogenic materials, in growth factor delivery and as scaffolds to heal tissue (Liang and Kiick 2014). Depending on the application, heparin-containing hydrogels have many desired properties, including anticoagulant activity, binding to growth factors as well as anti-angiogenic and apoptotic activities (He et al. 2019). Main advantages of naturally derived heparin hydrogels are biocompatibility, nontoxicity, good gelation conditions and they're relatively inexpensive. On the other hand, biohybrid hydrogels consisting of heparin and synthetic polymers allow precise control over their mechanical and chemical properties (Liang and Kiick 2014). The disadvantage of heparin hydrogels is that heparin can possibly lose its bioactivity or even break down during the production. It is also possible that heparin hydrogels can interact with the components of the blood, which is to be avoided (He et al. 2019).

Heparin hydrogels can be classified based on their size. The biggest of them, macro-hydrogels, are primarily used for pulmonary or oral delivery. They are difficult and inefficient to product, which limits their use in medical treatment. Hydrogels can also be injected into the body, and they turn from an amorphous form into their hydrogel form with specific physiological conditions. These so-called injectable hydrogels are used in tissue engineering, combining growth factors, tumor chemotherapy and wound healing. They can also mimic the ECM as a drug delivery system for postoperative chemotherapy, regeneration of damaged tissue and cell delivery carrier. The smallest of the heparin hydrogels are nanogels, which are a type of micro-hydrogels. They are a diverse and versatile group of nanoparticles, which can be used for example in drug delivery (He et al. 2019). It is also possible to consider nanogels to be nanocarriers, since they share many same qualities, such as nano-scale size, high drug loading capacity, high cellular uptake capability and enhanced penetration of tissue barriers (Dai Hai Nguyen et al. 2011). Their small size offers more surface area for drug conjugation and drug-loaded nanogels can be administrated through inhalation, parenteral or topical administration. They can also be injected into the blood either for systemic release or into a specific site. Nanogels are often used in cancer cell-targeting delivery, where they carry anti-fibrotic and anti-cancer agents, and in gene delivery. Nanogels can be prepared using microfluidic technology, chemical crosslinking, or hydrogen bonding (He et al. 2019).

Chemically crosslinked hydrogels have covalent bonds between chains, and they are the most used strategy to produce heparin hydrogels. For instance, they include photo-crosslinked hydrogels, which utilize photo-induced polymerization in their production. There are also Michael-type addition reaction, which bonds different molecules together for example by grafting ene or thiol groups onto heparin molecules, and rapid click-chemistry method, both of which are especially popular with injectable hydrogel formation. Other crosslinking methods include amide bond formation reaction and free radical polymerization, which are used to introduce vinyl groups to heparin, and enzyme-coupled reaction (He et al. 2019; Liang and Kiick 2014). The biggest advantages of chemically crosslinked heparin hydrogels are their bigger resistance to mechanical forces compared to physically crosslinked hydrogels and it is also easy to modify their mechanical properties to whichever application (Liang and Kiick 2014). There are also some disadvantages with chemically crosslinked hydrogels, such as the structure and activity of heparin might be destroyed during production, its production often requires the use of toxic chemicals, and it requires strict reaction conditions during production (He et al. 2019).

Physically crosslinked heparin hydrogels originate either from the entanglements between dynamic macromolecules or because of the noncovalent interactions between the polymer chains. Many heparin binding proteins, such as antithrombin III, heparin interacting protein and human platelet factor 4, can be used in the non-covalent formation of the hydrogel networks. In addition to proteins and peptides, growth factors can also be crosslinked with heparin hydrogels (Liang and Kiick 2014). Some examples about assembling physically crosslinked hydrogels are using electrostatic interaction, hydrophobic interaction, and hydrogen bonding. Advantages of these hydrogels are that they stay clear from toxic crosslinking reagents, which could otherwise lower bioactivity, and they allow injecting because it is simple to re-establish noncovalent bonds. Additionally, the production is easier and simpler compared to chemically crosslinked hydrogels and heparin usually maintains its structure and activity. Some of their disadvantages are the bad mechanical properties of hydrogels and that heparin could be eluted from the hydrogel (He et al. 2019; Liang and Kiick 2014).

Stimuli-responsive heparin-based hydrogels go through significant physical or chemical changes as a reaction to environmental stimuli. Some of these changes include swelling, shrinking, and degradation of the hydrogel or sol-gel phase transition, which is the change from colloid liquid to gel. By controlling the environmental stimuli, such as pH, enzymes, temperature, and light, it is possible to have a triggered release of drugs or biomolecules from the hydrogel. An example of stimuli-responsive heparin-based hydrogels is glutathione-responsive hydrogel. Glutathione (GSH) is a tripeptide, which is found in cellular compartments or tumor microenvironments. There are hydrogels with reduction-sensitive bonds like disulfide bonds, which degrade in the environment with a lot of glutathione. This makes targeted intracellular delivery of drugs possible (Liang and Kiick 2014).

As an example of stimuli-responsive hydrogels, Dai Hai Nguyen et al. developed a disulfide-crosslinked heparin-Pluronic (DHP) nanogel to improve the stability, redox receptivity, and the effectiveness of intracellular protein delivery. Pluronic is an amphiphilic thermogelling block copolymer, which undergoes a phase transition from liquid to gel at certain temperature. It inhibits the multidrug resistant activity occurring on the surface of cancer cells, but it has a poor capacity to encapsulate biological drugs. Conjugating Pluronic to heparin has many advantages, as heparin can interact better with many proteins and its biological activities are well known. Since physically assembled nanogels would be too fragile to secure the loaded proteins in the blood or support their release, disulfide linkages were used to stabilize them. First the thiolated heparin-Pluronic conjugate was self-assembled and oxidized to create a disulfide-crosslinked nanogel network under diluted aqueous circumstances. These linkages promote the selective release of drugs under the reductive conditions of the cytosol, which contains more glutathione than the ECM. Then positively charged RNase A, which has specific binding affinity to heparin and forms condensed non-covalent networks with it, was encapsulated into the DHP-nanogel. The DHP-nanogels displayed decreased hydrodynamic size, higher encapsulation rate, and enhanced release depending on the GSH concentration as the disulfide bonds of the nanogel were cleaved in the intracellular redox environment. The DHP-nanogels showed reduction-sensitive release that took advantage of biological activities of heparin (Dai Hai Nguyen et al. 2011).

4.2 Heparin-based hydrogels for tissue regeneration

Hydrogels are used a lot for tissue regeneration since they have many advantages, such as adjustable mechanical properties with alternating polymer content and rate of crosslinking. Modifying them with naturally derived polymers improves cell and tissue interactions and using biomolecules, like heparin, allows them to regulate endogenous growth factors (Nilasaroya, Kop, and Morrison 2021). Heparin binds to many growth factors and cytokines due to its high negative charge, so heparin-modified hydrogels or biodegradable scaffolds can interact with and retain growth factors and then release them slowly in a controlled way (Laner-Plamberger et al. 2021). Heparin-functionalized hydrogel scaffolds incorporate heparin as part of the building blocks of the hydrogel via copolymerization techniques, which have been shown to enhance the attachment and spreading of cells. However, to mimic the ability of heparin to sequester growth factors and release them in the ECM, heparin must be accessible for the enzymatic degradation from the biomaterials (Nilasaroya et al. 2021).

A lot of heparin-binding proteins have actually heparan sulfate as their endogenous ligand, but since it is not as easily available as heparin, and due to their similar structure, heparin is used instead (Hachim et al. 2019; Mitchell et al. 2021). Many growth factors have domains with high binding affinity for heparin. Common pro-angiogenic growth factors for both drug delivery and tissue

engineering are fibroblast growth factor-2 (FGF-2), vascular endothelial growth factor (VEGF), and platelet-derived growth factor (PDGF). Heparin-mediated delivery of FGF-2, which promotes angiogenesis and cell proliferation, has been utilized in many delivery systems, such as in nanoparticles and hydrogels. As a result of strong binding between heparin and FGF-2, the complex has very high loading efficiency and heparin protects FGF-2 from proteolytic degradation and maintains continuous release for long time. Heparin enhances the signaling efficacy of FGF-2 by helping it to bind better to FGF-receptors, acting as its co-factor. Heparin-based materials have also been used to deliver bone morphogenetic proteins (BMP), which are part of the TGF-β superfamily and are clinically used to promote cartilage and bone formation. Using BMPs in high doses leads to negative side effects, so different delivery systems have been created to avoid them. According to studies, presence of heparin in hydrogels increases the loading capability for BMP-2 and prolongs its sustained release. It is also possible to load multiple different growth factors at the same time for coordinated and synergic delivery. For example, using a heparin-based hydrogel to deliver both FGF-2 and VEGF has better cell proliferation and vascularization effects than a single delivery would have (Hachim et al. 2019).

Poly-(2-hydroxyethyl methacrylate) (PHEMA) hydrogels have many applications. Modifying non-anticoagulant heparin with methacrylate pendant groups allows it to promote covalent incorporation into the hydrogel. The affinity of growth factors naturally binding to heparin to heparin-functionalized hydrogels, their bioactivation and response on cell proliferation were assessed on human mesenchymal stromal cell (MSC) cultured on the hydrogel surface. Surface roughness and microporosity was presented to hydrogels during 3D scaffold formation due to the presence of heparin. PHEMA-heparin hydrogels could retain FGF-2 and present it to MSC cells as a result of binding to heparin. Hydrogels incorporated with both heparin and FGF-2 supported MSC growth for longer time compared to hydrogels with only one of them, because heparin stabilizing and protecting FGF-2 has a synergistic effect to long-term cell growth. Adjusting the sulfation patterns of heparin also affected cell morphogenesis and fate in MSC delivery matrix. Heparin-functionalized biomaterials are known to promote the reversible conjugation of heparin-binding growth factors associated with various wound healing processes. For example, PEG and poly-(vinyl alcohol) based heparin hydrogels have been studied for delivering FGF-2 and VEGF to promote angiogenesis and transforming growth factor-β1 (TGF-β1) to activate myofibroblast differentiation essential for the tissue regeneration. On the other hand, heparinized collagen scaffolds storing insulin-like growth factor-2 improved the bioactivity of FGF-2 to decrease the activity of TGF-β1 mediated cardiac fibrosis. These examples demonstrate the importance of heparin as an anchoring molecule for the growth factors and allow their consequent release from the hydrogels (Nilasaroya et al. 2021).

Besides growth factors, heparin can also bind to cytokines and chemokines. These interactions can be used to regulate cell recruitment, inflammation, and tissue remodeling. For example, a heparin-based hydrogel maintained sustained delivery of interleukine-4 (IL-4) a few weeks, which led to more efficient polarization of macrophages to the regulatory type than IL-4 alone. On the other hand, chemokine affinity of heparin can be utilized in removing chemokines instead of delivering them. For instance, a heparin-based hydrogel that was used as a wound-dressing improved wound healing in mice by sequestering the inflammatory cytokines from the injury (Hachim et al. 2019).

Wenbo et al. made a chitosan-heparin hydrogel for controlled release of chemokine called stromal cell-derived factor-1α (SDF-1α). SDF-1α and its receptor are expressed in various tissues and especially among stem cells. Delivering SDF-1α to the trauma site accelerates the regeneration in many tissues and it is a potential approach for intrauterine adhesion healing. Chitosan-heparin based hydrogels are stable and form noncovalent cross-linking. Similar to heparin, chitosan is a natural polysaccharide with some similar properties to heparin, such as biocompatibility and degradability. The hydrogel was formed by blending an acid chitosan solution and a basic heparin solution, which resulted in neutralized liquid with crosslinked chitosan and heparin by electrostatic attraction and physical entanglement. The polymer had a very porous structure so it could present both carrying capability and transporting route for small diffusible molecules. In comparison to artificial polymers used in hydrogels, like PEG, chitosan-heparin polymer demonstrated higher affinity to growth factors, cytokines, and chemokines due to their functional groups. According to study, the sol precursor of the hydrogel was turned to gel at physiological temperature in 15 minutes. Using western blot assay and immunofluorescence staining showed that endogenous stem cells were recruited to the injury site with increasing quality in 7 days, which promoted the recovery of the wound. The group of rats, who were treated with SDF-1α hydrogel, displayed a longer time attracting effect for stem cells in comparison to other groups. According to Masson trichrome staining and H&E staining, the endometrial recovery was significant with less collagen deposition. Immunohistochemical staining showed clear epithelium recovery and repair, and neovascularization in endometrium was characterized. Function recovery assay proved the healing effect of SDF-1α hydrogel. It is likely that the heparin dissociated from the hydrogel functioned as a healing agent as well and accelerated the healing and regeneration (Wenbo et al. 2020).

Wound dressings are supposed to preserve a humid environment around the wound and encourage the course of re-epithelialization, absorb the excess exudates from the injury site, tolerate the exchange of gaseous fluids, and shield against the infections while being non-adherent to the wound. Hydrogels are ideal for this purpose because of their properties. Their high inherent water content and swelling properties for absorbing exudates from the wound site allow them to maintain moist surroundings to the wound and protect it from possible dehydration. Their capacity to absorb a lot of biological fluids without being dissolved gives them physical qualities comparable to natural

soft tissues. Since it is known that making growth factors bind to heparin extends their half-lives, protects them from degradation and increases their bioactivity, a heparin-based hydrogel sheet consisting of thiolated heparin and diacrylated PEG was assembled by UV-photopolymerization and epidermal growth factor (EGF) was stored into it. The efficacy of the hydrogel on healing the skin wounds was observed both *in vitro* and *in vivo.* EGF accelerates and enhances wound healing by stimulating cell proliferation, collagen deposition, and migration of keratinocytes. Heparin-based hydrogels were able to carry and display EGF more effectively than normal PEG-hydrogel sheet, because of the interactions between heparin and EGF molecules. It was observed that coating wound sites with any hydrogel sheets provided faster closure of the wounds than the group treated only with EGF without the hydrogel, but ultimately EGF-loaded heparin-based hydrogel sheet advanced the closure of the wound significantly faster than any other group. Histological and immunological tests on EGF-loaded heparin-hydrogels also showed accelerated formation of the granulation tissue and capillaries, as well as epithelialization, hair follicle formation, oriented collagen layer formation and no macrophages that would indicate inflammation. In other words, EGF-loaded heparin-based hydrogel not only promotes faster the wound closure but also promotes the wound remodeling back to normal skin tissue, thus it would be suitable for wound dressing applications (Goh et al. 2016).

5. CONCLUSION

There are many different applications for heparin derivatives, including cancer therapy, gene therapy and wound healing. Properties and safety of many heparin derivatives are studied in various tests and some of the derivatives are already in clinical testing. Many biomolecules and transfer of genes are important in treating diseases and cancer, but due to their poor survival in the extracellular environments, more nanocarrier systems need to be created and heparin has shown to be an ideal candidate for them due to its interactions with various biomolecules.

There are still issues in treating cancer with heparin derivatives. The antitumor action of heparin derivatives is specific for certain tumors so only subgroups of patients can be treated with specific heparin derivatives, which additionally creates a challenge to identify molecular determinants associated with different types of cancer. For example, gs heparin drug Roneparstat showed clear antimetastatic activity against cancer cells, which produced both heparanase and P-selectin ligands, but was ineffective against cancer cells, which only produced selectin ligands (Lanzi and Cassinelli 2018).

Many treatments in general show improvements with only a part of patients, since there's a lot of heterogeneity among patients. Precision therapeutics are personalized interventions with improved therapeutic efficacy. Combining nanoparticles and precision medicine could advance both fields (Mitchell et al. 2021). Heparin derivatives, with their unique properties, have potential to be used in precision medicine, which still struggles with acquired drug resistance and intratumoral heterogeneity (Lanzi and Cassinelli 2018).

There is expected to be an increase in heparin integration within complex semi-synthetic materials in the future (Hachim et al. 2019). Additionally, heparin-based biomaterials have likely a more notable impact on treating many diseases and damaged tissues in the future (Liang and Kiick 2014). It is important and required to evaluate and control the quality of heparin-based nanoparticles. There should be more evaluative methods and indexes to guide their manufacturing (Yang et al. 2015). Because animal tissues are still the only sources for commercial heparin, the challenges of isolation and purification affect the safety and efficacy of it. There is also a risk for contamination with pathogens and other polysaccharides, like oversulfated chondroitin sulfate, so alternative sources for heparin should be identified. There are, of course, potential alternatives for heparin with similar properties, such as other glycosaminoglycans and synthetic oligosaccharides. Still, the use of heparin is the best alternative for biological delivery of certain proteins, like growth factors, due to their specific interactions (Lanzi and Cassinelli 2018).

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