



HHS Public Access

Author manuscript

Nano Today. Author manuscript; available in PMC 2020 October 01.

Published in final edited form as:

Nano Today. 2020 October ; 34: . doi:10.1016/j.nantod.2020.100898.

Overcoming negatively charged tissue barriers: Drug delivery using cationic peptides and proteins

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Abstract

Negatively charged tissues are ubiquitous in the human body and are associated with a number of common diseases yet remain an outstanding challenge for targeted drug delivery. While the anionic proteoglycans are critical for tissue structure and function, they make tissue matrix dense, conferring a high negative fixed charge density (FCD) that makes drug penetration through the tissue deep zones and drug delivery to resident cells extremely challenging. The high negative FCD of these tissues is now being utilized by taking advantage of electrostatic interactions to create positively charged multi-stage delivery methods that can sequentially penetrate through the full thickness of tissues, create a drug depot and target cells. After decades of work on attempting delivery using strong binding interactions, significant advances have recently been made using weak and reversible electrostatic interactions, a characteristic now considered essential to drug penetration and retention in negatively charged tissues. Here we discuss these advances using examples of negatively charged tissues (cartilage, meniscus, tendons and ligaments, nucleus pulposus, vitreous of eye, mucin, skin), and delve into how each of their structures, tissue matrix compositions and high negative FCDs create barriers to drug entry and explore how charge interactions are being used to overcome these barriers. We review work on tissue targeting cationic peptide and protein-based drug delivery, compare and contrast drug delivery designs, and also present examples of technologies that are entering clinical trials. We also present strategies on further enhancing drug retention within diseased tissues of lower FCD by using synergistic effects of short-range binding interactions like hydrophobic and H-bonds that stabilize long-range charge interactions. As electrostatic interactions are incorporated into design of drug delivery materials and used as a strategy to create properties that are reversible, tunable and dynamic, bio-electroceuticals are becoming an exciting new direction of research and clinical work.

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Author contributions

A. Vedadghavami and C. Zhang contributed to writing, review and editing of the manuscript. A. G. Bajpayee contributed to writing, review and editing, supervision and funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

Keywords

Targeted drug delivery; Negatively charged tissues; Electro-diffusive transport; Electrostatic charge interactions; Cationic drug carriers; Cell penetrating peptides; Cationic protein drug carriers

Introduction

The human body contains a multitude of negatively charged tissues that are dense and avascular and remain an outstanding challenge in the field of targeted drug delivery. While traditional delivery methods can bring drugs close to affected organs, intra-tissue drug penetration to reach cellular target sites is still ineffective. Examples of such tissues include cartilage, meniscus and ligaments of the knee, ankle, shoulder and hip joints etc., intervertebral discs (IVDs) in the spine, mucosal membrane of the gastrointestinal (GI) tract, vitreous humor of the eye, and skin (Fig. 1). Their degeneration is associated with several common diseases such as osteoarthritis (OA) [1] and gout [2] (synovial joints), spondylitis and cervical pain (spine) [3], inflammatory bowel disease (IBD) (GI tract) [4], and macular and retinal degeneration (eye) [5], which affect millions of people worldwide. There exist chemical entities, biologics and gene therapies that can inhibit or reverse tissue degeneration but only if they are able to reach their intra-tissue and intra-cellular sites in sufficient doses; their clinical application is limited by a lack of effective delivery systems that can enable drugs to penetrate through full tissue thickness to reach their cell targets and retain there to provide sustained drug release. Many of these diseases thus remain untreated. The avascular nature of the tissues makes them inaccessible to systemically delivered drugs [6]. Local drug delivery methods like intra-articular (IA) injection for synovial joint tissues, epidural injection for targeting IVDs, sub-acromial injection for shoulder bursa and intra-vitreous injection for back of the eye also remain inadequate; the dense extracellular matrix (ECM) and high density of negatively charged groups hinder entry and transport of drugs which clear out via lymphatics or vasculature before reaching the required intra-tissue therapeutic index. For example, corticosteroid injections used to treat local pain and inflammation have half-lives of only a few hours, necessitating frequent injections of high drug doses [6,7].

The complex ECM of such avascular tissues consists of a dense meshwork of collagen fibrils, proteoglycans (PGs), containing highly negatively charged glycosaminoglycan (GAG) chains, and cells, which create a barrier to entry for most drugs or drug carriers. For example, cartilage has a complex meshwork of type II collagen, which is densely packed with aggrecans, presenting substantial steric hindrance to penetration of therapeutic molecules. The meniscus has similar ECM structure to cartilage but a lower fixed charge density (FCD) (20 % aggrecan content versus 35 % in cartilage by dry tissue weight) [8]. The nucleus pulposus (NP) of IVDs is mostly composed of negatively charged PG gel held together loosely by a sparse network of type II collagen fibrils. The NP is surrounded by a tough fibrous coating of annulus fibrosus (AF) containing circumferentially aligned collagen making it difficult for drugs injected epidurally to penetrate via passive diffusion [9]. Similar issues plague drug delivery to the back of the eye as passive diffusion of drug in vitreous humor is inefficient, suffering from off-target effects and lacking sustained drug delivery

[10]. The negatively charged gastric mucosa also poses a hindrance to solute penetration making oral drug delivery challenging [11]. Similarly, the skin's stratum corneum (SC) and the viable epidermis-dermis (VED) containing densely packed cells, lipids, collagen, elastin, hyaluronic acid (HA) and GAGs hinder transdermal drug delivery [12]. Table 1 presents GAG content and estimated average negative FCD in some tissues.

Electro-diffusive transport of cationic solutes in negatively charged tissues

This high negative FCD of tissues can be utilized by modifying therapeutics to add optimally charged cationic domains such that the electrostatic interactions can enhance their transport, uptake and retention rather than hindering them [13,14]. Local administration of cationic carriers to negatively charged tissues results in a sharp increase in their concentration by a Donnan partitioning factor of K^+ (K^+C versus C at the administration site) [13], due to internal electrical fields exerted by the negative FCD of the tissue. Conversely, negatively charged solutes are repelled from the tissue resulting in a downward partitioning from concentration of C at the administration site to K^-C at the tissue interface while neutral solutes that are not sterically hindered can maintain the same concentration at both tissue interface and administration site [13]. Thus, the elevated concentration of positively charged solutes at the tissue interface results in steeper concentration gradients and faster intra-cartilage diffusion rates compared to both neutral and negatively charged counterparts [13] (Fig. 2A (i)). It was recently shown that by using electrostatic interactions, the intra-cartilage uptake of cationic drugs or their carriers increased by 100–400 times as compared to their neutral versions [14–18]. Consequently, the cationic carriers can reach their intra-tissue therapeutic index faster, ensuring an appropriate biological response before getting cleared from the administration site. Recent research efforts have focused on using strong specific binding mechanisms to increase the residence time of drug carriers inside tissues [19–22]. However, the ability of strong binding interactions to promote transport through tissues is paradoxical; while binding enhances retention, it consequently hinders diffusive transport slowing down the penetration of carriers as they would get trapped in the surface of the tissue before reaching deeper zone targets [13,14]. The weak and reversible binding nature of electrostatic interactions is indeed an essential characteristic that enables drug carriers to unbind after their initial binding with negatively charged groups, find another binding site, and continue this process until they diffuse through the full tissue thickness (Fig. 2A (ii)). It is the high negative FCD of such tissues that greatly increases the intra-tissue residence time of cationic solutes despite their weak binding. Several of the diseases affecting negatively charged tissues result in degeneration of tissue matrix, thereby inducing some GAG loss and a reduction in the tissue negative FCD. In these cases, charge-based binding of carriers can be further stabilized with the remaining GAGs by synergistically using short-range hydrophobic interactions and hydrogen bonds [14] (Fig. 2B).

Cationic peptide and protein-based drug carriers

Proteins have gained attention as safe natural biomolecules that can replace synthetic polymers applied in drug formulations [23]. Protein based drug delivery systems offer several advantages including biodegradation, biocompatibility and possibility of surface modification for targeted drug delivery [23]. Cell penetrating peptides (CPPs), which are

an increasing number of cationic therapeutics that are entering the clinical trial stage and are high-lighted in this article.

Here we discuss selected negatively charged, avascular tissues, and delve into how each of their structures, tissue matrix compositions, and high negative FCDs create barriers to drug entry. We explore how charge interactions are being used to overcome these barriers using cationic peptide and protein-based drug carrier systems. We review work on tissue targeting charge-based peptide and protein drug delivery, compare and contrast drug delivery designs, and also present examples of technologies that are entering clinical trials.

Musculoskeletal tissues

Musculoskeletal disorders impose an ongoing clinical challenge by affecting a wide variety of musculoskeletal tissue systems including articular cartilage, synovial joint and synovium, ligaments/tendons, meniscus and intervertebral discs (Fig. 3A–E).

Articular cartilage

Articular cartilage is a connective tissue which acts as a low friction gliding surface for articulation and distribution of mechanical loading across the joint [47]. Its avascular matrix comprises of a collagen II network (50–60 % tissue dry weight), filled with densely packed 300 MDa aggrecans (35 % dry weight) and a low density of chondrocytes (<5 % dry weight). Each aggrecan comprises of a central hyaluronan chain to which hundreds of 2–3 MDa aggrecan monomers are bound and contain negatively charged sulfated GAG chains (mainly chondroitin sulfate (80 %) followed by keratan sulfate (5–20 %)) [48,49] that are spaced only 2–4 nm apart along the monomer core protein. The density of these bottle brush structured aggrecans increases with depth into cartilage, reducing the effective pore size and restricting solute permeability and diffusion [13]. These negatively charged groups create a high FCD inside cartilage (–170 mM, Table 1) that provides the necessary hydration, swelling pressure and compressive stiffness, which are key to tissue function. The collagen II fibrils are aligned parallel to the surface in the articulating superficial zone, randomly oriented in the middle zone enabling load distribution and perpendicular to the subchondral bone in the deep zone (Fig. 3A). Degradation of articular cartilage can lead to a variety of joint degenerative disorders; OA is one of the most common forms affecting over 250 million people worldwide [50,51]. OA onset results in gradual loss of GAGs thus depleting the tissue's FCD and compromising matrix integrity. While many potential disease modifying OA drugs (DMOADs) have been identified, none of them have passed clinical trials due to poor cartilage targeting and off-target side-effects [6]. Currently, direct IA injections to affected joints are the primary route for delivering pain and inflammation relievers, however, a majority of the drug is rapidly cleared from the joint space through the lymphatic system and capillaries (for example, mean half-lives of NSAIDs in synovial fluid (SF) are only 1–4 h) [6], therefore requiring multiple injections of high drug doses which cause systemic toxicity. This is further complicated by the significant steric hindrance presented by tissue matrix that prevents penetration of therapeutics. Since a majority of chondrocytes reside in the middle and deep zones of cartilage, drug delivery to chondrocytes remains challenging.

Bajpayee and Grodzinsky showed that the high negative FCD of cartilage can be used for converting cartilage from a barrier to drug transport into a drug depot by modifying drugs or their carriers with optimally charged cationic domains such that the weak-reversible nature of electrostatic interactions can enhance their intra-cartilage transport, uptake, and retention [13,15,35,52]. Using short length cationic peptide carrier motifs, Vedadghavami et al. recently showed that there exists an optimal net positive charge to deliver a drug of given size to a tissue of known FCD that will result in rapid penetration through the full thickness of cartilage before a majority of it is cleared from the joint space, and can enable highest intra-cartilage uptake and long-term retention [14]. Using cationic peptides of varying net charge, they showed that intra-cartilage uptake increased with increasing net charge of peptides to +14 but dropped as charge increased further due to stronger binding interactions that hindered peptide penetrability and uptake [14]. Optimal net positive charge on the carrier is chosen to enable weak and reversible binding with the intra-tissue negatively charged groups, which is critical to allow for the drug and its carrier to penetrate through full tissue thickness and not get stuck in the tissue's superficial zones. Despite weak binding, the high negative FCD of aggrecan associated GAGs inside cartilage greatly increases the residence time of optimally charged cationic drug carriers [13,14]. Similarly, the cationic glycoprotein, Avidin, due to its optimal net size (<10 nm hydrodynamic diameter) and charge (between +6 and +20) [15] was shown to penetrate through full thickness of rabbit cartilage following IA injection (Fig. 4A (i)) [53], resulting in a high intra-cartilage uptake ratio of 180 (implying 180x higher concentration of Avidin inside cartilage than surrounding fluid at equilibration) and was still found to be present through the full thickness of cartilage two weeks following its IA administration in a rabbit anterior cruciate ligament transection (ACLT) model of post-traumatic OA (Fig. 4A (ii)) [16]. Avidin was covalently conjugated with 4 moles of Dexamethasone (Av-Dex) using its four biotin binding sites [54] and administered in a single low dose IA injection one week following ACLT in a rabbit model [16]. Av-Dex suppressed injury induced joint inflammation, synovitis, incidence of osteophyte formation and restored trabecular properties significantly better than free Dex (Fig. 4A(iii)) [16]. Recently, a multi-arm Avidin (mAv) nano-construct comprising of four 8-arm PEGs with high drug loading content (28 sites for covalent conjugation of drugs) was developed that can enable intra-cartilage delivery of a broad array of small molecule OA drugs to chondrocytes [55,237]. mAv was conjugated to Dex (mAv-Dex) using a combination of releasable ester linkers derived from succinic (SA), phthalic (PA) and dimethylglutaric anhydride (GA) in 2:1:1 molar ratio that provided a sustained Dex release over two weeks (Fig. 4B (i)). Consequently, mAv-Dex rescued IL-1 induced GAG loss significantly greater than free Dex (Fig. 4B (ii)).

Different classes of cationic carriers such as Poly-beta amino esters [56] and Poly(amidoamine) (PAMAM) dendrimers [35] were later developed for enhancing drug penetration and retention in cartilage. 6th generation PAMAM dendrimer was PEGylated at 45 % of its amine sites to minimize cytotoxicity (Gen 6 45 % PEG). Gen 6 45 % PEG conjugated to insulin like growth factor 1 (IGF-1) (Gen 6 45 % PEG-IGF-1) provided superior intra-cartilage penetration and retention using charge interactions between the remaining cationic amine groups and cartilage GAGs while free IGF-1 had completely cleared out of the tissue within 6 days post IA injection in rats (Fig. 4C (i)). Gen 6 45 %

PEG-IGF-1 reduced cartilage area of degradation from 24 % in the untreated group to only 8 % while free IGF-1 failed to significantly suppress cartilage loss compared to untreated ACLT group 4 weeks post-surgery (Fig. 4C (ii)). An electrostatic based self-assembled complex formed by sequentially mixing IGF-1 with negatively charged polyglutamic acid and 5.8 kDa positively charged polyarginine, was shown to be retained in the joint space for 4 weeks after IA injection in a rat OA model [57]. Tokunou et al. developed a fusion protein consisting of IGF-1 and a cationic heparin binding (HB) peptide sequence allowing selective binding to cartilage GAGs and chondrocytes [58]. The HB-IGF-1 fusion protein showed cartilage targeting via specific binding with heparan sulfate GAGs and dominant non-specific electrostatic binding with chondroitin sulfate GAGs [59]. HB-IGF-1 was retained within rat articular cartilage for 8 days post IA injection while there was no sign of free IGF-1 present at two days after injection [60]. Additionally, HB-IGF-1 significantly reduced surface cartilage loss compared to unmodified IGF-1 in meniscus transected rats upon weekly IA injection for three weeks [60]. Inagawa et al. studied the effect of varying chain lengths of polyarginines on their transport in cartilage [61]. They found that the uptake increased within increasing number of arginine up to 8 (R8) after which intra-cartilage accumulation declined. Also, through enzymatic degradation of different types of cartilage GAGs, they determined that chondroitin sulfates were the main source of binding interaction with R8. Bajpayee and co-workers recently studied the effects of net positive charge, type of cationic residue (arginine or lysine) and hydrophobic moieties on cartilage penetration and retention of cationic peptide carriers of same size [14]. They noted that arginine-rich sequences had higher uptake and retention in cartilage compared to lysine-rich sequences due to stronger binding affinity of arginine to negatively charged GAGs. Interestingly, they observed that even after depleting 50 %–90 % of the total intra-cartilage GAGs, arginine-rich sequences still had high intra-cartilage uptake and retention while lysine-rich sequences did not, suggesting the presence of other interactions, likely H-bonds and hydrophobicity that stabilize charge-based binding within cartilage tissue. This can be attributed partly to the chemistry of the guanidinium head-group on arginine residues that form stable bidentate hydrogen bonds with polarizable oxo-anions such as the sulfates in aggrecan GAGs. Additionally, guanidinium cations can form thermodynamically stable (weakly) *like-charge pairs* in water, where a combination of dispersion and cavitation forces from the medium can overwhelm the coulombic repulsion. Because of this, arginine moieties are capable of binding more strongly (due to synergistic effects of charge, H-bond and hydrophobic interactions) and co-operatively (due to formation of like-charge pair between arginine) with negatively charged GAGs compared to lysine moieties, which instead exhibit mutual coulombic repulsion. They also showed that the addition of hydrophobic moiety to lysine-rich sequence significantly enhanced its intra-cartilage uptake and retention in both healthy and arthritic cartilage, further emphasizing the advantages of combining synergistic effects of short-range hydrophobic interactions and H-bonds in stabilizing intra-tissue charge-based binding of drugs or their carriers, which is especially relevant for targeting mid to late stage arthritic tissues that have lost some GAGs and thus have lower negative FCD [14].

Targeting collagen 11 network has also been explored for enhancing drug specificity and retention within articular cartilage [19,20]. Rothenfluh et al. discovered WYRGRL sequence with specific binding affinity to collagen 11 in cartilage, which was later conjugated to

dexamethasone (Dex) through hydrolysable ester linkers [19,20]. This prodrug retained in cartilage deep zones for extended time periods and showed enhanced efficacy compared to free drug, a strategy that can be useful for targeting later stages of OA when the majority of GAGs have been lost [20]. Hu et al. developed 1,4,7,10-tetraacetic acid amide (DOTAM) nanoparticles with improved cartilage affinity by incorporating both WYRGRL for specific collagen 11 targeting (TP) and primary amines for charge-based binding to deliver Cathepsin D inhibitor (pepstatin A) in an acid-stimulated GAG depleted porcine cartilage culture model [62,63] (Fig. 4D (i)). DOTAM nanoparticles decorated with TP (DOTAM-TP) showed enhanced retention in mice knee joints 48 h post IA injection compared to DOTAM conjugated with a nonbinding control peptide sequence (Fig. 4D (ii)). DOTAM-TP was also highly localized in cartilage 24 h post administration as shown in Fig. 4D (iii). DOTAM comprising of one collagen 11 binding peptide and two amine groups inhibited GAG loss by 41 % within 24 h while the DOTAM with only three collagen 11 binding peptides provided 34 % GAG loss suppression. By 48 h, when a significant extent of GAGs from cartilage was depleted, DOTAM with three collagen 11 binding domains was still effective in suppressing GAG loss by 18 %. The system provides flexibility to adjust the relative extent of weak charge-based binding and strong specific binding with collagen 11 depending on the degree of GAG loss from cartilage for effective targeting.

Chondrocyte affinity has also been used for targeted drug delivery to cartilage [64,65]. Chondrocyte affinity peptide (CAP) with sequence DWRV11PPRPSA identified using phage display effectively delivered Hif-2a siRNA to chondrocytes when conjugated to PEI [66]. CAP when conjugated to PEG-PAMAM enhanced penetration into cartilage, joint retention and chondrocyte uptake [67]. Also, VLTTGLPALISW1RRRHRRHC (p5RHH), a positively charged peptide derivative of melittin [68] was used to suppress NF- κ B pathway using siRNA delivery to chondrocytes in a mechanically induced mouse injury model [69]. Additionally, it was noted that p5RHH-siRNA complexes could penetrate up to 700 μ m of cartilage and suppress Interleukin 1 beta (IL-1 β) activated p65 in cytokine induced human cartilage culture for three weeks with only a single dose treatment [70]. Fusion protein consisting of cationic carrier PEP-1 and Glutaredoxin-1 (GRX-1), an anti-oxidant enzyme in homeostasis, was also shown to significantly suppress inflammatory markers compared to free GRX-1 [71].

Synovial joint and synovium

Since the synovial joint is subjected to rapid clearance, major research efforts have focused on developing large, non-penetrating, non-binding drug carriers that remain suspended in SF following IA injections and due to their large size, do not exit via the synovium lymphatics or vasculature. These carriers are most relevant when the target sites of the drug are mainly in the SF or synovial membrane, such as with drugs used for relieving pain and inflammation [72]. They are, however, not effective at stimulating disease modifying response in chondrocytes. Examples of such drug delivery systems include drug encapsulating microparticles [73], polymeric micelles [74], liposomes [75], aggregating hydrogels and peptides [76]. The HA in SF is not sulfated but its carboxylic acid groups impart some negative charge (Fig. 3B), which has also been used for enhancing retention of drugs inside the joint by using positively charged chitosan [77,78] or by making

nanoparticles cationic [79] so they can ionically crosslink with HA. The negative charge in SF can also hinder transport of cationic carriers into cartilage by competitive binding [80,81], however, the negative FCD of cartilage is significantly higher due to the presence of sulfated GAGs providing greater affinity for charged carriers (20 fold lower FCD, -8.8 mM compared to -170.0 mM, Table 1). Bajpayee et al. showed that Avidin, owing to its optimal net charge, rapidly diffused through the full thickness of rat and rabbit cartilage following IA injection resulting in high uptake and long-term retention despite the presence of SF and dynamic compression induced convective flow in the knee joints [53,82]. Recently, it was reported that the uptake of supercharged cationic green fluorescent proteins (S-GFP) from SF into cartilage reduced by 20–50 % than that from PBS depending on the net charge of S-GFP, yet high intra-cartilage mean uptakes of 5–20 were reported [52].

Strategies to target synovium have also been explored because of its pivotal role in joint inflammation and pain [72]. Mi et al. developed multiple cationic peptide sequences and studied their efficacy in transducing synovial fibroblasts [83]. The net positive charge of peptide was determined as an important factor in their internalization capability. For example, short length peptides with sequences PIRRRKCLRRLK (+8 charge) and RRQRRTSKLMKR (+7 charge) had superior transduction capability compared to KRIQRILSRNS (+4 charge). However, both peptides showed similar performance compared to their 12-mer polylysine or polyarginine counterparts. Therefore, it was concluded that although cationic charge is crucial, beyond a certain charge limit, factors like peptide conformation also influence peptide transduction efficacy. The same group later identified a specific synovium fibroblast binding sequence, SFHQFARATLAS (HAP-1) with the goal of targeted delivery of apoptotic agents to hyperplastic synovium [84]. HAP-1-liposome systems were also used to deliver an immunosuppressive peptide to inflamed joints [85]. Furthermore, HAP-1 liposomes conjugated with NF-KB-blocking peptide were shown to significantly suppress synovial inflammation [86]. Additionally, the unique cell markers present in newly formed blood vessels associated with angiogenesis in inflamed synovium have been used for targeting [87,88]. For example, peptide sequence CKSTHDRLC with binding affinity to synovial vascular endothelium was fused to anti-inflammatory cytokine interleukin 4 (IL-4) that effectively accumulated in the synovium providing enhanced retention and bioactivity [89].

Ligaments, tendons and meniscus

Ligaments and tendons have similar tissue structure, predominately comprising of type I collagen (about 75 % of dry weight) organized linearly to provide load bearing tensile strength [90] (Fig. 3C). Leucine-rich PG, decorin, helps tie adjacent collagen fibrils together, while aggrecans and versicans provide tissue viscoelasticity allowing fibrils to slide smoothly over each other [91] (Fig. 3C). Ligament and tendon injuries constitute 50 % of the total 33 million musculoskeletal injuries reported annually in the United States [92]. These tissues lack self-healing capability and surgical intervention is often required post injury. Meniscus is a fibrocartilaginous tissue located between tibia and femoral condyle, which is responsible for shock absorbance and force transmission [93] (Fig. 3D). Meniscus damage usually occurs as a result of traumatic injury or degenerative diseases. Meniscus dry weight is mainly comprised of collagen (75 %) followed by aggrecan GAGs (17 %) [8]. Its

collagen network is predominantly comprised of collagen I (90 %) oriented circumferentially while collagen II fibrils are only found in the inner one-third of meniscus (Fig. 3D) [8]. Aggrecans are the major type of PGs present mostly in the inner two-third of the tissue [8]. Tears in meniscus, especially in the inner two-third avascular zone suffer from poor self-healing capability [94]. Historically, meniscus tissue was considered to be a functionless vestigial structure, which would be excised (or completely removed) after damage [93]. However, the importance of meniscus as a joint stabilizer and shock absorber is now well recognized and its preservation is essential to maintain normal knee biomechanics. Various suturing techniques for repairing torn ligaments [95] and menisci [96], substitution using autologous tissues [97] or allografts [98], biologically inert or cell seeded biomaterials [99,100] and 3D printed tissue engineering strategies [101,102] are being explored.

In case of partial tears, the presence of negatively charged GAGs in these tissues can be utilized for delivering anti-catabolic drugs and growth factors via IA injected charged carriers (average FCD of -51.5 mM for meniscus, -6.6 mM for tendon and -17.9 mM for ligament, Table 1). Distribution of positively charged Avidin post IA injection in rat knee joints revealed that both uptake and retention of Avidin in joint tissues correlated strongly with tissue GAG content [82], which decreased in the order of cartilage, meniscus, ligament and patellar tendon. Charge interactions can be coupled with motifs with binding affinity to specific matrix constituents within the tissue to further enhance tissue specificity.

Intervertebral disc (IVD)

The IVD is avascular and functions as a ligament holding the vertebrae together, a shock absorber mitigating stresses on the body, and a pivot point allowing for spine flexibility. The IVD is composed of the NP, the gelatinous center region of the IVD, the AF, the tensile connective tissue that restricts the NP using circumferential (hoop) stresses, and the cartilaginous endplate which connects the NP and AF to the vertebra. The NP is composed of negatively charged PG gel held together loosely by a sparse network of type II collagen fibrils [103] (Fig. 3E). Degeneration of the IVD, particularly in the NP is often characterized by inflammation and a loss of GAGs resulting in decreased disc height and function. This leads to lower back pain, which is one of the leading causes of disability worldwide [104]. For IVD regeneration, a wide range of therapeutics including anti-catabolic, pro-anabolic factors and chemo-attractants that can stimulate the resident cells and recruit endogenous progenitors are under consideration [105–107]. The avascular nature of this tissue makes it challenging for systemically administered drugs to reach their target cells inside the NP. Therefore, local intra-discal injection to deliver drugs directly into the NP is a suitable approach especially since the tissue is well encapsulated by endplates and AF. Drugs injected in this manner, however, suffer from rapid and wide diffusion outside the injection site resulting in short lived benefits while causing systemic toxicity [105,106]. Toxicity concerns are further aggravated when potent biologics, gene editing/silencing vectors, or proteolytic enzymes are used. Current in vitro and in vivo work relies on frequent drug dosing, which is clinically infeasible. It is, therefore, critical to develop effective approaches for enhancing intra-NP drug residence time and cell targeting.

Drug delivery systems under investigation include both nano- and micron-sized polymeric particles such as injectable nanoparticles [108], microspheres [9], hydrogels [109], and tissue engineering scaffolds [110] but have shown short intradiscal residence time of few hours requiring the need for multiple injections. Other systems consist of synthetic biodegradable polymers like poly(lactide) (PLA), poly(glycolide) (PGA), poly(ϵ -caprolactone) (PCL) for their tunable properties and minimal immunogenicity [111]. The high negative FCD of the NP (Average FCD of -138.2 mM, Table 1) also presents an opportunity for enhancing the residence time of cationic drug depots enabling sustained therapeutic benefits [13]. Recently, chitosan functionalized Pluronic-based nanoparticles were used for intra-discal delivery of chymopapain; electrostatic binding within the NP kept the delivery of encapsulated enzyme localized to the injection site at least over 24 h [108]. Wagner et al. showed that cationic Avidin grafted dextran can enable a month long intra-discal residence time [112].

Clinical translation and future direction

Musculoskeletal tissues remain an outstanding challenge in the field of drug delivery due to their avascularity and complex architecture comprising of densely packed negatively charged groups (Table 1). There is a critical need for targeted drug delivery systems to pave way for treating burdensome diseases like OA. Recently, intra-joint sustained release formulations of triamcinolone encapsulated within micron sized poly (lactic-co-glycolic acid) (PLGA) particles (Flexion Therapeutics, Burlington, MA, USA) were approved by the FDA for OA pain and inflammation [113,114], but such systems fail to target cells inside cartilage and to elicit long-term disease modifying effects to restore joint function. Use of optimally charged cationic protein and peptide carriers is a promising direction that can enable safe, effective delivery of drugs to cell and matrix targets residing deep within tissues, thereby eliciting disease modifying effects as well as facilitating long-term symptomatic pain and inflammation relief. Significant success has been demonstrated using pre-clinical models in recent years and these approaches can now be considered for moving to clinical trial stage to translate newly discovered drugs or to repurpose those that previously failed OA trials due to lack of cartilage targeting and associated systemic toxicity. Finally, while the vast amount of data in this field has been generated using cartilage, more work is foreseen in using these approaches for other tissues like meniscus, ligaments, tendons and IVD.

Eye

Over 250 million people are affected by vision impairment worldwide, mostly prevalent among population over 50 years old [115]. The anterior part of the eye is composed of cornea, pupil and the lens, which function to collect and focus the light while the posterior segment is comprised of the retina, vitreous, macula and optical nerve which together function to detect light [116] (Fig. 5A). Dry eye, glaucoma and cataract are common diseases affecting the anterior tissues. In contrast, the posterior chamber is threatened by age-related macular degeneration (AMD), retinitis pigmentosa and diabetic retinopathy [117]. If left untreated, most of these diseases can ultimately lead to vision loss and blindness. Despite the need for treatment of ocular diseases, there is currently no cure available for AMD [118]. Ocular drug delivery remains challenging due to the presence of

several physiological and anatomical barriers that are normally responsible for protecting the eye against foreign materials [119].

95 % of the total drug administered topically is lost through nasolacrimal drainage, tear turnover and conjunctival blood circulation [119]. Consequently, corneal penetration of the remaining dose of drug is impaired by the epithelium [120]. The vitreous humor accounts for 80 % of eye volume, stretching from lens to retina, also acts as a major barrier to diffusion of drug delivery systems. It is predominantly comprised of hydrophilic negatively charged 2–4 MDa HA chains (Fig. 5A) and to a lesser degree chondroitin and heparin sulfate (average FCD of -0.6 mM, Table 1). While cationic carriers have been shown to effectively penetrate through the negatively charged pores of the corneal epithelium, transport of positively charged carriers could be hindered in the vitreous as they become immobilized through charge interactions [121,122]. Käs Dorf et al. studied the effects of net positive charge on diffusion of cationic drug carriers in the vitreous humor using oligopeptides of same size but varying charge (between +7 and +23) and showed that there exists a threshold of positive charge on a carrier for effective diffusion through the vitreous humor (up to +15) [46]. Further increase in net charge to +19 and +23 resulted in significant suppression and complete blocking of transport of cationic peptides in the vitreous humor. Various types of cationic carriers including naturally derived peptides such as penetratin [123,124], TAT [125,126] and PEP-1 [127,128] as well as synthetic peptides like lysine [129] or arginine-rich [130] sequences have been used for enhancing drug bioavailability and ocular penetration of drugs or their carriers. For example, penetratin, TAT and polyarginine (R8) have shown 87.5, 31.8 and 16.3 times higher corneal permeation respectively compared to negatively charged control, poly(serine)₈ in vitro [131]. Additionally, in vivo studies showed that topically administered penetratin rapidly diffused to the posterior section of the eye within 10 min and was retained for more than 6 h while the negatively charged polyserine peptide completely cleared out of the eyeball within 2 h. The superior performance of penetratin compared to other cationic peptides was mainly attributed to its amphipathic nature. Similar results were observed by Jiang et al., who showed that penetratin's permeability through rabbit cornea increased significantly when hydrophilic residues in its sequence were replaced with hydrophobic tryptophan [123]. Penetratin-PAMAM systems for DNA plasmid delivery were later developed that retained for more than 8 h in rat retina following topical administration and reached the posterior segment of the eye through the sclera [124]. Tai et al. developed penetratin-PAMAM antisense oligonucleotide (ASO) for ocular tumor gene silencing for topical use that reduced expression of target gene by 2-fold compared to PAMAM-ASO with no penetratin [132]. Following this, topical application of penetratin functionalized nanoparticles were shown to effectively deliver siRNA and suppress target protein expression in retinoblastoma mouse models [133].

TAT is another naturally derived peptide used for enhancing ocular penetration of nanoparticles. Zhang et al. [134] formed anti-angiogenesis delivery systems through a fusion protein system comprising of TAT and endostatin that could target retina and elicit anti-angiogenesis response. Similarly, TAT has been coupled with human acidic fibroblast growth factor (TAT-aFGF) and with inhibitory peptide sequence against rat mitochondrial μ -

calpain (responsible for photoreceptor cell death) and shown to rapidly target retina and elicit therapeutic response for at least 6–8 h when topically administered in rats [135,136].

The Kumar-Singh group designed a peptide for ocular delivery (POD), GGG(ARKKAAKA)₄, which is based on the heparin binding sequence (XBBBXXBX)_n as the concentration of heparin in retina increases with disease progression [137,138]. POD showed high uptake in human embryonic retinal cells, strongly bound with cornea and sclera within 45 minutes of topical administration (Fig. 5B (i)) and transduced 85 % of neural retina within 2 h post intra-vitreous injection (Fig. 5B (ii)) in mice [137]. Subretinal injection of PEGylated POD nanoparticles for DNA delivery were shown to increase gene expression by 215 times compared to native DNA. An updated version of PEG-POD with a reducible disulfide linkage (PEG-SS-POD) conjugated with vascular endothelial growth factor (VEGF) receptor 1 transgene was later developed which allowed dissociation of PEG in the tissue matrix for enhanced intra-cellular uptake of the POD modified nanoparticle [139]. Subretinal injection of this reducible nanoparticle showed 50 % reduction of neovascular growth in a choroidal neovascularization (CNV) mouse model compared to the control group. Vasconcelos et al. modified PLGA-PEG nanoparticles with POD for corneal delivery of flurbiprofen (FB) with the goal of suppressing inflammatory response in surgical intervention of cataract [140]. Briefly, FB encapsulated PLGA particles were conjugated to bifunctional Maleimide-PEG-amine through EDC/NHS chemistry. Then POD-sulfhydryl (POD-SH) groups were incorporated on PLGA-PEG particles through reaction with Maleimide groups to form POD modified PLGA-PEG-FB nanoparticles (POD-PLGA-PEG-FB) (Fig. 5C). POD-PLGA-PEG-FB formulation significantly prevented inflammation compared to commercial eye drops (Ocufer®), PLGA-PEG-FB and TAT-PLGA-PEG-FB (Fig. 5D) in a sodium arachidonate (SA) inflammation induced rabbit model due to its higher retention and uptake in corneal epithelium. Furthermore, de Cogan et al. examined ocular penetration of synthetic arginine-rich peptide with sequence RRRRRR forming complexes with anti-VEGF drugs [141]. Upon topical administration, the complexes diffused to anterior segment of rat eyes within 6 min, which were as efficient as the intra-vitreous injection of the anti-VEGF agent in CNV mouse models.

Clinical translation and future direction

As discussed, a significant body of research exists in using charge-based peptides for enhancing drug targeting to cornea, the vitreous humor and retina of eye thereby improving their residence times and therapeutic efficacy even via the topical route. XG-102 or Brimapitide, (Xigen, Epalinges, Switzerland), a TAT coupled dextrogyre peptide that selectively inhibits the c-Jun N-terminal kinase previously shown to effectively suppress ocular inflammation in rat uveitis model [42] became the first peptide to be subconjunctivally administered clinically to bypass the epithelium for treating post-operative ocular inflammation [142]. In a phase II randomized, double blind, controlled, multi-center trial of 145 patients in Europe, a single subconjunctival injection of Brimapitide immediately following an ocular surgery was shown to be non-inferior to Dex eye drops given 4 times daily for 21 days [143]. An outstanding challenge, however, is reaching the target sites in the posterior region of the eye, including macula at the far end of the back of the eye, which is the site for a number of diseases such as AMD, retinitis pigmentosa and diabetic retinopathy.

Currently intra-vitreous injections are required to target the macula since topical delivery of drugs/drug carriers suffer from low bioavailability and getting stuck in the vitreous humor before reaching the macula, likely due to strong electrostatic binding within the vitreous. It should be possible to design optimally charged drug carriers with weak and reversible binding properties as discussed above in the example of musculoskeletal tissues. These carriers would have net charge that is weak enough so as not to hinder their diffusion through the vitreous yet strong enough to have long residence times and not exit quickly through lymphatics.

Mucosal membrane of the GI tract

Oral drug delivery is the most desired method for drug administration due to its convenient, non-invasive and patient-friendly merits. However, oral delivery of biologics, proteins, peptides and other macromolecular drugs is limited due to their lack of stability from enzymatic degradation in the acidic environment of GI tract and their inability to transport across the mucus-epithelial barrier [144]. Mucus is a viscoelastic and dynamic gel layer consisting primarily of 90–98 % water, 2–5 % (w/v) crosslinked and entangled mucin fibers, and small amounts of lipids and proteins [144]. Mucins are high molecular weight glycoproteins consisting of two domains: PTS (proline, threonine, serine) domain and a naked hydrophobic region [145]. As Fig. 6A and B show, the heavily glycosylated (e.g. sialic acid and fucose) repeats in the PTS domain make mucin highly negatively charged (typically 20–30 carbohydrates per 100 amino acids) contributing to the majority of non-specific hydrogen bonding and electrostatic interactions (Average FCD of -4.4 mM, Table 1) [145–147]. The non-glycosylated (naked) protein domains are hydrophobic and rich in cysteine containing disulfide bonds that enable crosslinking of monomers to form mucin gel. The mucin gel network provides a 500–700 μm protective barrier with a heterogeneous mesh pore size ranging from 50–1800 nm depending on its location along the GI tract and the state of disease, providing steric hindrance to solute diffusive transport [148,149]. The thickness of mucus layer in the intestinal lumen of adolescents with IBD was found to be about three times thinner than the healthy mucin layer [150]. Typically, GI mucus consists of an outer loosely adherent luminal layer and a firmly adherent layer (Fig. 6A) that attaches to a dense array of negatively charged glycoproteins, termed glycocalyx that are present on the apical surface of the tightly connected epithelial cells, which form the next barrier layer after the mucin gel network [145].

Electro-diffusive transport of solutes across mucosal barrier

Transport rates of solutes across the porcine GI mucus have been shown to significantly decrease with increasing particle size from 20 to 500 nm [151]. In addition, mucus permeability highly depends on charge-based binding interactions between the solute of given net charge and the negatively charged mucus. Positively charged nanoparticles are immobilized within the mucus due to electrostatic interaction but negatively charged or neutral nanoparticles can penetrate through mucus easily. Crater et al. demonstrated that high degree of interaction between amine-modified particles and negatively charged mucin fibers significantly reduced particle transport rates whereas carboxylate-modified particles showed five-fold higher transport rates [152]. A recent study, however, showed that the

penetration ability depends on binding strengths of solutes with mucus constituents; cationic dextrans that weakly adhered to mucin gels with low to intermediate binding affinities were shown to penetrate through the native mucus at equally high efficiencies as uncharged, non-mucoadhesive molecules [153]. Li et al. demonstrated that the transport behavior through the mucosal barrier cannot be predicted by solute net charge alone and that its spatial distribution is also a critical parameter that modulates its transport [45]. A zwitterionic block peptide (AKAKAKAKAKAEAEAEAEAE) containing cationic and anionic segments arranged in separate blocks exhibited a discernable concentration peak at the entry of mucin barrier due to Donnan's effect resulting in a high transport rate, which was greater than that in the mucin-free (buffer control) group. In contrast, a peptide with similar amino acids as the block peptide but arranged in an alternating sequence, as opposed to separate blocks (AKAEAKAEAKAEAKAEAKAE) showed a monotonically descending concentration profile at the entry of mucin barrier (Fig. 6C) [45]. Additionally, unlike the block peptide, the alternate peptide had similar transport rates in mucin and buffer control groups [45]. These differences between transport profiles of the block and alternate peptides show that the mucin barrier can distinguish the spatial configuration of charged residue on a nanometer length scale [45]. The study also noted that the transport rate was higher for zwitterionic peptides (block or alternating) compared to purely anionic or cationic peptides. This is important as viruses and native proteins also contain combinations of positive and negative surface charges that may modulate their transport across the mucosal barrier. While the positive charge on the particle can lead to enhanced transport rate owing to Donnan partitioning, it can also cause binding to mucins, which can slow down solute diffusive transport through the mucin layer. Therefore, both these mechanisms may interact in complex ways to produce the selective permeability effect observed with cationic, anionic, block and alternating peptides in this study. These concepts can be used for multi-functionalizing drug and gene carriers with positive and negative charges to modulate their transport and targeting in the mucus barrier [45,154].

Mucoadhesive nanoparticles

Since the hydrophobic regions in mucin can trap hydrophobic particles, use of hydrophilic 'mucus-inert' coating has been proven to be a successful strategy to minimize particle entrapment within the mucus layer [155]. For example, PEGylation of tobramycin [156] and lipid polymer nanoparticles [157] shield their positive charge and hydrophobicity respectively, thereby enhancing their permeability across mucus. Multiple studies have suggested using mucolytic agents to break disulfide bridges and cleave the mucin glycoprotein cross-linked network as a strategy to overcome the mucus barrier, but this disruption is not immediately reversible raising serious concerns [158,159]. A few reports have reported the issue of back-diffusion following elimination of particles in the loosely adherent mucus layer due to its rapid turnover and clearance by peristaltic forces, which results in a drug concentration gradient in the opposite direction of penetration [158]. Therefore, contrary to 'mucus-inert' particles, mucoadhesive strategy has also been utilized by using positively charged and thiolated chitosan-modified particles [21,160] or by co-administering drugs with polyarginine [161]. Jain et al. designed cationic 'layersomes', containing alternate layers of anionic poly (acrylic acid) and cationic poly-amine hydrochloride synthesized using layer by layer method, which increased oral bioavailability

of encapsulated doxorubicin by 5.9 folds compared to free drug significantly inhibiting tumor growth [162]. Mucoadhesive nanoparticles, however, have the tendency to disrupt mucosal barriers, which can increase the risk of exposure to bacteria and foreign particles [163].

Researchers have also developed approaches for localized targeting of mucus at the site of inflammation [164–167]. Zhang et al. developed anionic self-assembling hydrogels comprising of amphiphilic ascorbyl palmitate (AP) capable of cleaving under inflammatory environment for local targeting of the inflamed intestine to treat IBD [164]. They noted that the negative charge of the hydrogel allowed selective binding to the inflamed colon epithelium which had a lower FCD due to loss of mucin layer and was retained up to 12 h post a single enema in a colitis mouse model. The hydrogel was used to deliver Dex which provided higher therapeutic efficacy than the free drug.

Epithelium targeting mucus penetrating nanoparticles

While negatively charged or net neutral nanoparticles could rapidly penetrate through the mucus with minimal binding interactions and back-diffusion, their uptake by epithelial cells is expected to be much lower compared to positively charged particles. For example, insulin when conjugated with cationic CPP resulted in 6–8 times higher intestinal absorption compared to normal insulin in Caco-2 cell monolayer [40]. Although the mechanism of CPP mediated cell uptake remains unclear, one of the well-accepted hypotheses is that the initial contact and binding between the negatively charged cell membrane and positively charged CPPs triggers both endocytosis and micropinocytosis pathways [168]. PEGylation can help shield some cationic charge to minimize binding with mucus while using the residual charge for enhancing targeting and uptake in the epithelial cells. For example, CPP functionalized PEG-modified mesostructured silica nanoparticles were shown to penetrate through the mucus, enhancing cellular uptake of encapsulated recombinant growth hormone (RGH) by 4.9 fold compared to free RGH in vivo [169]. Recently, a novel nano-delivery system capable of changing its zeta potential from neutral or negative charge to positive charge while penetrating through the mucus barrier was developed to overcome the issue of binding with mucus while enabling epithelial cell targeting and uptake [158,170,171]. Wang et al. modified quantum dots doped hollow mesoporous silica nanocarriers (HSQN) with cationic CPPs and concealed these peptides using a hydrophilic succinylated casein (SCN) layer (Fig. 7A) [172]. SCN layer digested by trypsin enabled zeta potential of nanoparticles to markedly change from around -20 mV to $+40$ mV. In vivo oral administration of this dual-modified charge-changing nanoparticle HSQN@CPP@SCN encapsulating paclitaxel and quercetin revealed that SCN coating enabled nanocarriers to easily penetrate through the rat mucus; presence of trypsin in gastric juices degraded SCN exposing CPPs enabling 8-fold higher plasma concentration of encapsulated paclitaxel compared to oral delivery of free drug, Taxol®. A similar approach was used by coating dissociable thiolated polymer (PSH) on CPP functionalized mesoporous silica nanoparticles (MSN) [173]. PSH worked as a mucoadhesive agent and gradually dissociated from nanoparticles as it penetrated through the mucus leaving a strong positive surface charge ($+31.9$ mV) on nanoparticles [173]. Ex vivo imaging of whole-body distribution exhibited higher concentration of MSN-CPP-PSH nanoparticles than that of MSN or MSN-CPP nanoparticles remaining in isolated intestines

(Fig. 7B). As such, MSN coated particles resulted in 6.8-fold increase of relative bioavailability compared to unfunctionalized silica particles. Huang and colleagues utilized the charge-reversal strategy by adding negatively charged phosphoserine (Pho) to their octa-arginine (R8) functionalized PLGA nanoparticles (P-R8-Pho), imparting virus like neutral charged surface (-2.39 mV) which rapidly penetrated through the mucus (Fig. 7C) [174]. The surface anchored anionic Pho was hydrolyzed by intestinal alkaline phosphatase (IAP), which reversed its zeta potential to positive ($+7.37$ mV), exposing cationic R8 peptide that enabled effective CPP-mediated cellular uptake and transepithelial transport in a Caco-2/E12 coculture epithelium model [174]. P-R8-Pho also effectively induced a maximal reduction in blood glucose by 32 % in fasted diabetic rats, while oral administration of insulin showed no hypoglycemic effect.

Clinical translation and future direction

Recently, orally ingestible macro gastro-retentive devices and microneedles have gained attention to improve oral drug bioavailability. Traverso and colleagues designed an oral capsule that unfolds into a star shape after reaching the stomach that due to its large expandable size, cannot pass through the stomach resulting in long residence time of 3–4 weeks during which desired drugs loaded in polymeric arms of the star are released into the stomach [175–177]. Overtime, the star breaks down into small pieces due to presence of dissolvable elements made of enteric polymers that remain stable in the acidic gastric environment but dissolve in near neutral or basic pH (as is in the duodenum) enabling easy passage out of the body [175–178]. The team's orally ingestible microneedles demonstrated significant improvements in insulin bioavailability compared to the subcutaneous route, and is especially promising for the delivery of large sized protein and other biomolecule drugs [179]. In summary, mucosal administration of biologics and protein drugs is desirable especially because it can bypass the hepatic first-pass metabolism thereby increasing the bioavailability of drugs. Its use, however, is limited by poor drug absorption and drug degradation in the acidic GI tract, which is particularly concerning for biologics including peptides, proteins, anti-bodies and nucleic acids. As a result, biologics are still administered using injections. In recent years, a few of mucus penetrating formulations have translated to clinical trials. For example, Rybelsus (Novo Nordisk, Bagsværd, Denmark) became the first FDA approved oral tablet, a co-formulation of Glucagon like peptide 1 receptor antagonist (GLP-1 RA) with sodium N-[8-(2-hydroxybenzoyl) amino] caprylate (SNAC), for treatment of Type 2 diabetes in the United States [180]. SNAC protects the peptide against enzymatic degradation by exerting buffering action to neutralize local acidic environment near the site of tablet erosion, thereby decreasing the efficacy of digestive enzymes and protecting the peptide from pepsin degradation. SNAC also efficiently inserts into the plasma membrane of gastric epithelium affecting membrane fluidity but not affecting the tight junctions. This enhances transcellular absorption of the non-covalently associated GLP-1 RA allowing it to pass through the gastric epithelium into the systemic circulation [181]. An inhibitor of P-glycoprotein (P-gp), Encequidar (Athenex Inc., Buffalo, NY, USA), has been shown to enhance oral absorption of a wide range of anticancer drugs and is in clinical trials [182]. P-gp is an active transport protein expressed in intestinal epithelium that can pump back orally administered anti-cancer drugs that are P-gp substrates. Oraxol, an oral tablet which is a combination of oral paclitaxel with Encequidar was shown to achieve similar paclitaxel

exposures compared to widely used intravenous weekly dosing regimen [183,184]. This formulation is now completing a phase 3 clinical trial ([NCT02594371](#)) in metastatic breast tumors [185].

Skin

The transdermal route offers another non-invasive and convenient method for drug delivery. Abundant vasculature and capillary beds present in the dermis layer of skin enable direct transport of drugs or drug carriers into the circulation escaping the enterohepatic circulation, thereby providing a more reliable clinical response. However, effective methods to transport across the SC and VED barriers are needed to make transdermal route viable for drug delivery [186]. The outermost layer of skin is the SC composed of 40 % water by weight, 40 % protein (mostly keratin) and 15–20 % lipid predominately triglycerides, cholesterol, fatty acids and phospholipids [186]. The tightly packed corneocytes (keratin enriched dead keratinocytes) in SC are embedded in a lipid matrix giving it a “bricks in mortar” like structure [187] (Fig. 8A). The intracellular space is packed with semi-crystalline α - and β -keratin. Human SC is 10–15 μm thick containing 10–25 corneocyte layers that are oriented approximately parallel to the skin surface [186]. The intercellular lipids are arranged orderly in layers (lamellae) making the SC barrier tight and dense enough to prevent penetration of macromolecules over 500 Da [186]. The next layer below SC is viable epidermis (50–100 μm thick) which is a stratified, squamous and keratinizing epithelium containing melanocytes, Langerhans cells and Merkel cells [188]. Dermis (1–2 mm thick) is composed of ample levels of collagens, elastin, HA and other GAGs (Fig. 8A). HA is one of the most abundant components of the ECM of dermis (~0.5 mg/g wet weight) and more than 50 % of total HA in the body is found in skin (average FCD of –2.5 mM, Table 1) [189]. In addition to vasculature and capillary beds, dermis contains nerve tissues and appendages such as hair follicle, hair shaft, sebaceous glands and sweat glands. VED are relatively hydrophilic compared to SC and serve as an important permeation barrier for transdermal delivery [190]. Hypodermis is a fatty connective tissue layer carrying sensory nerves and blood vessels [188]. A wide range of methods have been designed to permeate the skin by physically disrupting the epidermis (iontophoresis, ultrasound, jet injection, microneedles, thermal ablation) or by using chemical permeating enhancers [186].

Peptide based transdermal delivery systems

Recently, CPPs have sparked considerable interest due to their ability to penetrate through the skin SC, which is the first barrier for transdermal drug delivery. The cationic charge from arginine and lysine residues in CPPs or CPP-drug conjugates enhances their binding to negatively charged cell membrane and ECM components like heparin sulfate, phospholipids and keratin ($pI = 4.0$ – 6.5) in the SC barrier [191–193]. This creates an electrical potential gradient generating inward pointing electric fields providing a driving force that plays a significant role in the translocation and penetration of CPPs into different layers of the skin [194].

Chang et al. showed that higher positively charged cyclopeptide TD-34 (ACSSKKS⁺KHCG) with bis-substituted lysine in N-5 and N-6 locations of TD-1 (ACSSSPSKHCG)

significantly enhanced transdermal permeability of insulin lowering blood glucose levels to about 26% of initial levels in diabetic rats in vivo [195]. A skin penetrating and cell entering (SPACE) peptide (AC-TGSTQHQ-CG) ethosome nanoparticle system was shown to enhance HA delivery to dermal layer by 5-fold compared to vehicle free HA delivery in PBS in vivo (Fig. 8B) [196]. The HA concentration was 1000-fold higher in the dermal layer under the local application site than that tested in the blood [196]. The same system was also shown to enhance transdermal siRNA delivery [197]. Kumar et al. demonstrated that SPACE, TD-1 and polyarginine (R7) significantly enhanced cyclosporine A (CsA) penetration into skin via the transcellular pathway mediated by their enhanced partitioning into keratin-rich corneocytes due to charge-based binding with keratin and CsA [198]. The three skin penetrating peptides did not alter skin lipid barrier and showed negligible effect on skin integrity and cytotoxicity. SPACE peptide was reported to be least toxic to keratinocytes and among the most effective at delivering CsA into the skin. Recently, TAT conjugated with aFGF showed enhanced epidermis penetration and enabled superior recovery of skin ulcers compared to free aFGF by depositing more organized collagen rich dermis in rat skin injury models (Fig. 8C) [199]. Catalase (CAT), an anti-oxidant enzyme, was fused with TAT and arginine-rich peptide (R9) to form TAT-CAT and R9-CAT fusion proteins that efficiently penetrated the epidermis as well as the dermis when sprayed on the animal skin [200]. The HIV-TAT peptide was also shown to enhance the transdermal delivery of NSAIDs [201].

Full-thickness skin penetrating peptides

The exact mechanisms of CPP transport across the transdermal barrier remain unknown but can happen through one of the following pathways, **a.** transcellular, **b.** intercellular, **c.** trans-appendage and **d.** pore-forming (Fig. 8A) [202,203]. While some research has ruled out transcellular route and endocytosis mechanism as the corneocytes in the SC of skin are non-viable [203,204], Rothbard et al. suggested that SC is still a metabolically active environment despite no viable cells [41]. This was corroborated by Hou et al. efforts that showed that macropinocytosis inhibitor, 5-(*N*-ethyl-*N*-isopropyl)-amiloride and the F-actin polymerization inhibitor, cytochalasin D, reduced transdermal delivery of arginine-rich CPPs and proteins conjugated to them [205]. CPPs can interact with lipids triggering the intercellular delivery pathway [204,206]. For example, polyarginine was shown to enhance transdermal permeability of FITC-labeled dextran by opening tight junctions in rabbit nasal epithelium [207,208]. A T2 peptide (LVGVFH) enhanced penetration of bacteriophage across porcine and mouse skin which was mediated by interactions with intercellular lipids of SC owing to the presence of histidine and lysine in the sequence [209]. A significant level of accumulation of CPP IMT-P8 (RRWRRWNRFNRRRCR), TAT and their peptide-protein fusion constructs inside the hair follicles after 24 h transdermal delivery have also indicated that trans-appendage mechanisms are at play [210]. Magainin (GIGKFLHSAKKFGKAFVGEIMNS), a peptide known to form pores in bacterial cell membranes has been shown to increase skin permeability by disrupting the SC lipid structure [211]. Magainin's charge has been modulated to electrostatically bind the molecule of interest for its effective delivery across the skin. For example, Magainin with a +2 charge at pH 7.4 enhanced transdermal penetration of negatively charged fluorescein by 35 fold; however, when its charge was neutralized by increasing the pH to 10, effects of electrostatic

interactions between magainin and fluorescein were removed which eliminated the enhanced permeability effect from Magainin [212].

For a long time, VED barrier was not considered in designing transdermal delivery systems; transporting across the SC was considered sufficient. Thus, the transdermal delivery of compounds was overestimated in experiments using epidermal membrane and underestimated in those using full thickness skin or in animal models [190,213]. Matrix properties of SC and VED barriers vary in composition, thickness, fixed charge distribution and hydrophobicity. For example, since the dermis has high water content, hydrophilic compounds can penetrate through the full thickness of skin and distribute throughout the dermis, while a lipophilic propoxur was found to get stuck in the SC and only partially penetrate the dermis [214,215]. Pepe et al. compared the transport behavior of microemulsions modified with primary amphipathic CPP (transportan), secondary amphipathic CPP (penetratin) and TAT in SC and VED, all of which rapidly penetrated through the SC but transportan demonstrated the highest penetration rate through the VED while TAT displayed the slowest, showing that amphiphilic properties also play a strong role in addition to positive charge [216]. Although microemulsion TAT showed the lowest penetration rate in VED barrier in Pepe et al.'s study [216], Zheng et al. showed that TAT enhanced the accumulation of aFGF in both dermis and subcutaneous tissues facilitating healing of deep tissue injury in skin [199]. Peptides that can transport across the VED can create drug depots in the dermis that can potentially find applications in wound healing of deep skin injuries and for stem cell therapies [217,218].

Clinical translation and future direction

A CPP based formulation that successfully entered phase II clinical trials is PsorBan, a cyclosporine – hepta-arginine conjugate (Cell Gate Inc., Redwood City, CA, USA) for topical treatment of psoriasis by transdermal delivery of CsA [37]. While phase IIa trials showed potential benefit in treating mild-to-moderate psoriasis without the adverse effects associated with systemic use of cyclosporine, subsequent trials were interrupted in 2003 as the release of the free drug was not rapid enough to compete with clearance [37]. Revance Therapeutics Inc., Newark, CA, USA, developed a topical gel, RT001 for treatment of crow's feet lines (wrinkles around the eyes) comprising of 150 kDa botulinum toxin type A (BoNTA) and a novel CPP which is a reverse sequence of basic residues of TAT, suggested to have improved transdermal penetrability [219,220]. The peptide electrostatically binds with BoNTA such that its PTDs (protein transduction domains) are directed outward to attach with cell membrane [219,220]. While phase II studies were promising, the topical gel preparation of RT001 did not achieve its co-primary endpoints in phase III clinical trials. An injectable formulation was later developed which is now in clinical trials [220].

Conclusión

Negatively charged PGs are critical for structure and function by providing hydration, swelling pressures, compressive stiffness and signal transduction. Large PGs like aggrecans in cartilage have numerous highly sulfated GAG side chains, which hold water and resist deformation owing to increased electrostatic repulsion between the negative charges as they

come closer to each other during compressive loading. Small PGs like decorin, biglycan, fibromodulin, lumican are closely associated with collagen fibrils and are believed to regulate collagen fibril diameter. At the cell membrane, PGs stabilize ligand-receptor binding triggering signaling complexes that regulate cell proliferation, migration, matrix adhesion and endocytosis. While critical for tissue function, these PGs make the tissue extremely dense conferring a high negative FCD (Table 1) and making intra-tissue drug delivery extremely challenging.

This high negative FCD, however, can be converted from being a challenge to an opportunity by utilizing the weak, reversible nature of electrostatic interactions by making drugs optimally positively charged. Mechanistic transport studies using peptides and proteins with different net charges across tissues of varying FCD (cartilage, vitreous of eye, mucin, skin) (Table 2) have shown that an optimal charge can be determined for a peptide carrier such to take advantage of Donnan partitioning induced enhanced transport such that the electrostatic interactions are weak enough for the carriers to rapidly get past the tissue superficial zones but strong enough to bind within the deep zones for long term retention. Other short-range binding interactions like hydrophobic and H-bonds can synergistically stabilize long-range charge interactions and thus can enhance drug retention within diseased tissues that have lost PGs and have lower negative FCD. A vast body of literature exists on charge mediated CPP based drug delivery to cells some of which are now in clinical trials. Multi-stage delivery systems that can sequentially penetrate the tissue and then target cells are under development.

These concepts provide rational methods of designing drug carriers that are tunable based on the net tissue FCD, state of disease, where target sites reside and the physico-chemical properties of the drug. As electrostatic interactions are incorporated into design of drug delivery materials and used as a strategy to create properties that are reversible, tunable and dynamic, bio-electroceuticals are becoming an exciting new direction of research and clinical work. Such smart materials promise tremendous impact by addressing the needs of an entire set of negatively charged tissues which are ubiquitous in the body, are associated with a large number of common diseases but have been largely overlooked primarily due to drug delivery challenges.

Acknowledgment

This work was supported by the United States Department of Defense through the Congressionally Directed Medical Research Programs (CDMRP) under contract W81XWH-17-1-0085, National Institutes of Health (NIH)R03 EB025903-1 and NIH TrailblazerR21 EB028385-01.

Biography



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Ambika Bajpayee is an Assistant Professor in the Department of Bioengineering at Northeastern University. Her interests include targeted drug delivery, bio-electrostatics, peptide and protein-based nanocarriers, and modeling of bio-transport and biomechanics with a specific focus on degenerative musculoskeletal diseases. She received her Masters and PhD in Mechanical Engineering at Massachusetts Institute of Technology (MIT) and also completed her post-doctoral work focused on developing devices for oral drug delivery to gastrointestinal tract with Prof. Robert Langer. Previously, she worked as a medical device engineer on development and FDA approval of orthopedic and dental implants. Ambika is a recipient of the US Department of Defense Discovery Award, National Institute of Health Trailblazer Award, MIT Women of Excellence and MIT Meredith Kamm Award in Mechanical Engineering.

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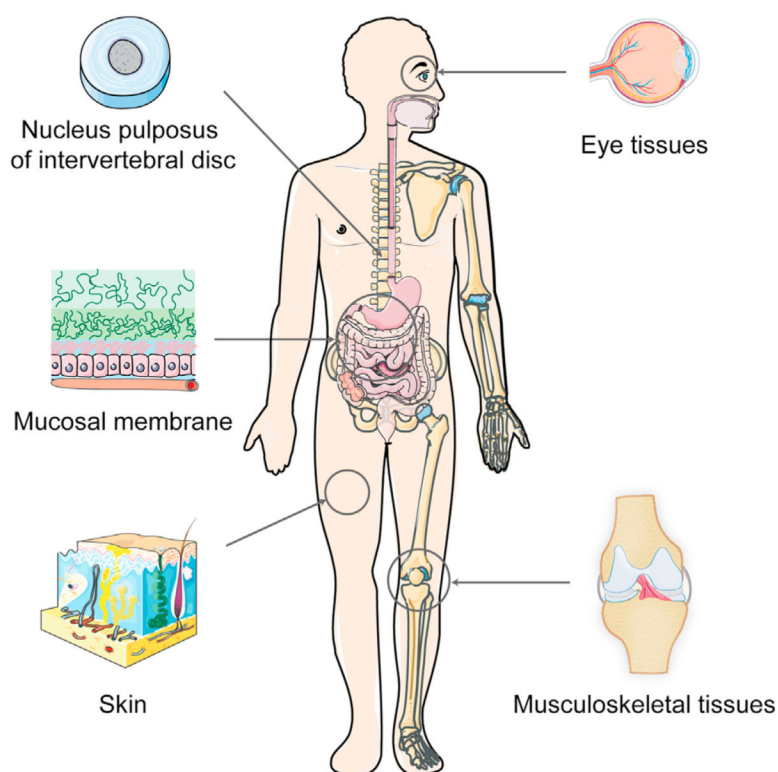


Fig. 1. Negatively charged tissues in the human body remain an outstanding challenge in the field of targeted drug delivery. Examples include musculoskeletal joints(shoulder, elbow, knee, hand and ankle) comprising of tissues like articular cartilage, synovial joint, ligaments, tendons and menisci; nucleus pulposus of the intervertebral disc; eye tissues like the cornea and vitreous humor; mucosal membrane; and skin comprising of multiple layers of negatively charged cells, collagen, proteoglycans and elastin. This high negative FCD can be used to enhance intra-tissue transport, uptake and binding of locally injected drugs or their carriers via electrostatic interactions by modifying them to contain optimally charged cationic domains.

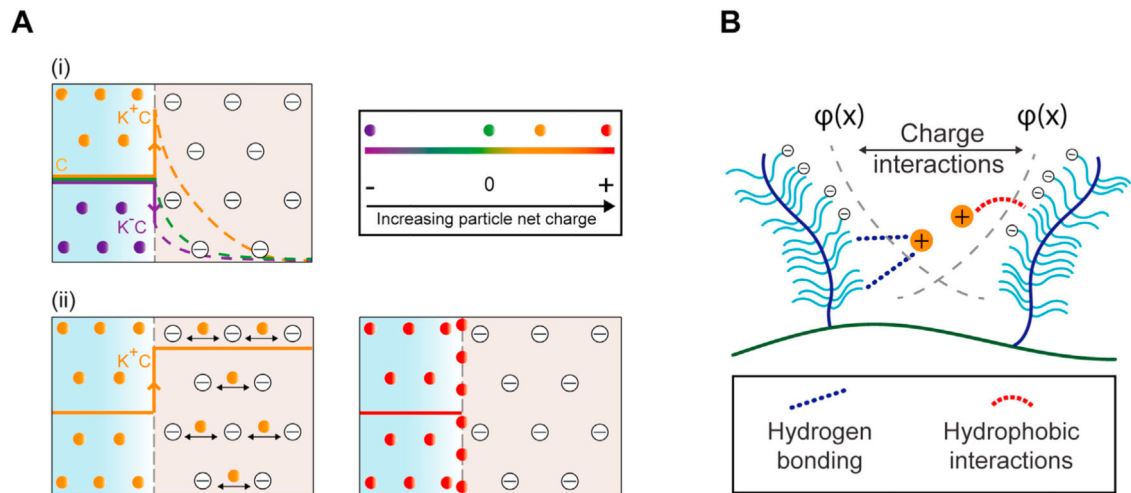


Fig. 2.

Electro-diffusive transport of cationic carriers in negatively charged tissues. **A.** (i) Concentration of positively charged carriers partitions upward from C to K⁺C at tissue interface enabling faster diffusion rates compared to their neutral counterpart. Conversely, concentration of negatively charged carriers partitions downward from C to K⁻C due to electrostatic repulsion resulting in slower intra-tissue diffusion rates. (ii) Optimally charged cationic carriers can penetrate through the full thickness of tissues owing to weak-reversible binding nature of charge interactions while carriers with too high positive charge get stuck within the tissue surface due to strong binding interactions. **B.** Incorporation of short-range effects such as from hydrophobic or hydrogen bonds can synergistically stabilize long-range charge-based binding of cationic carriers with their intra-tissue negatively charged binding sites. $\phi(x)$ denotes the electric potential exerted by fixed negatively charged groups as a function of distance, x inside the tissue.

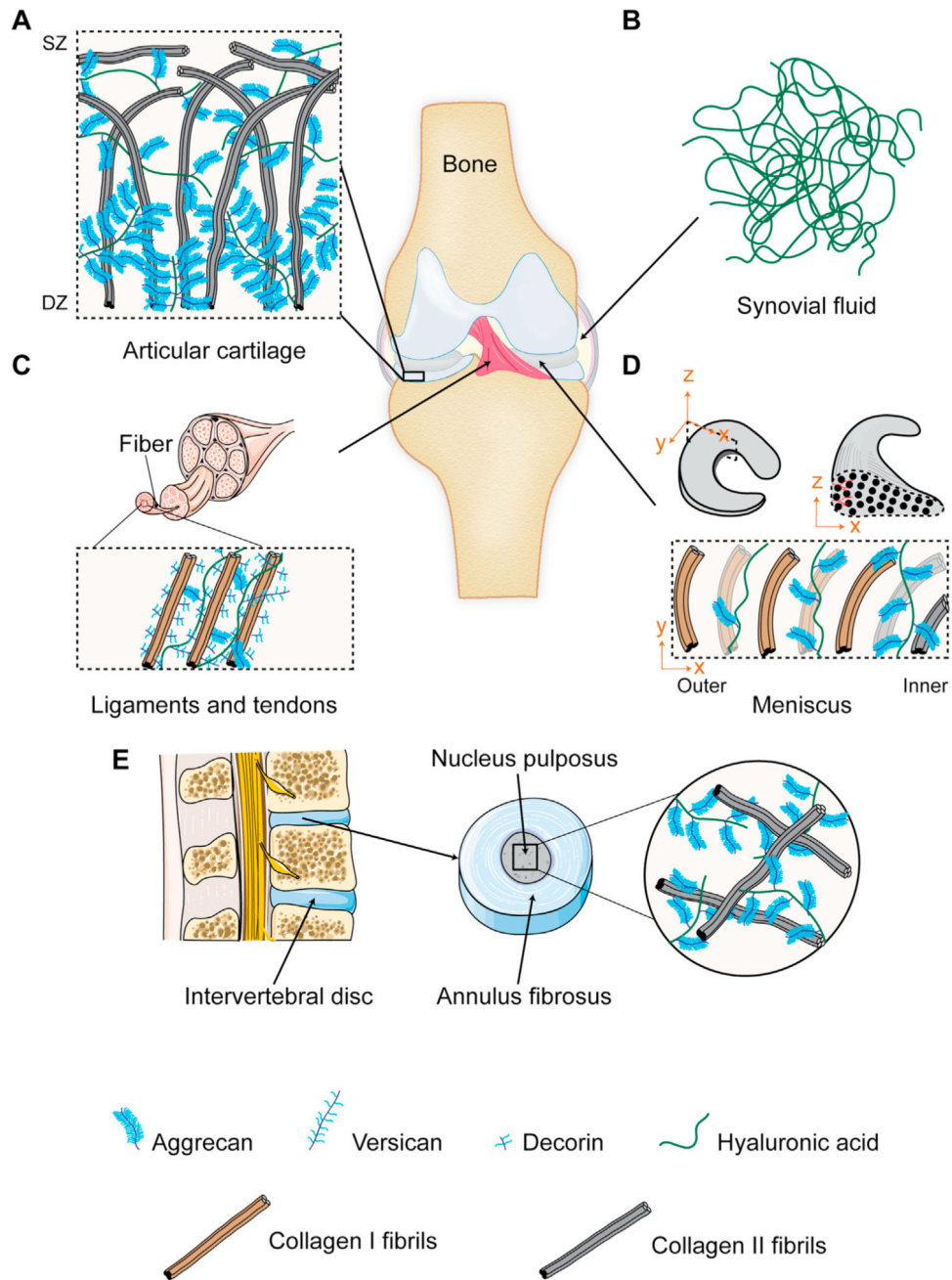


Fig. 3. Musculoskeletal joint tissues. **A.** Articular cartilage is comprised of a network of collagen II and a high density of aggrecans. Collagen fibrils are aligned parallel to the surface in the superficial zone (SZ) while arranged perpendicularly in the cartilage deep zone (DZ); aggrecan density increases from SZ to DZ. **B.** Synovial fluid is comprised of high molecular weight hyaluronic acid meshwork. **C.** Ligaments and tendons are comprised of linearly aligned collagen I fibrils containing decorin, aggrecans and versicans. **D.** Meniscus is comprised of circumferentially aligned collagen fibrils, mainly type I in the outer two-third region and a combination of types I and II in the inner region. Aggrecan density is highest in

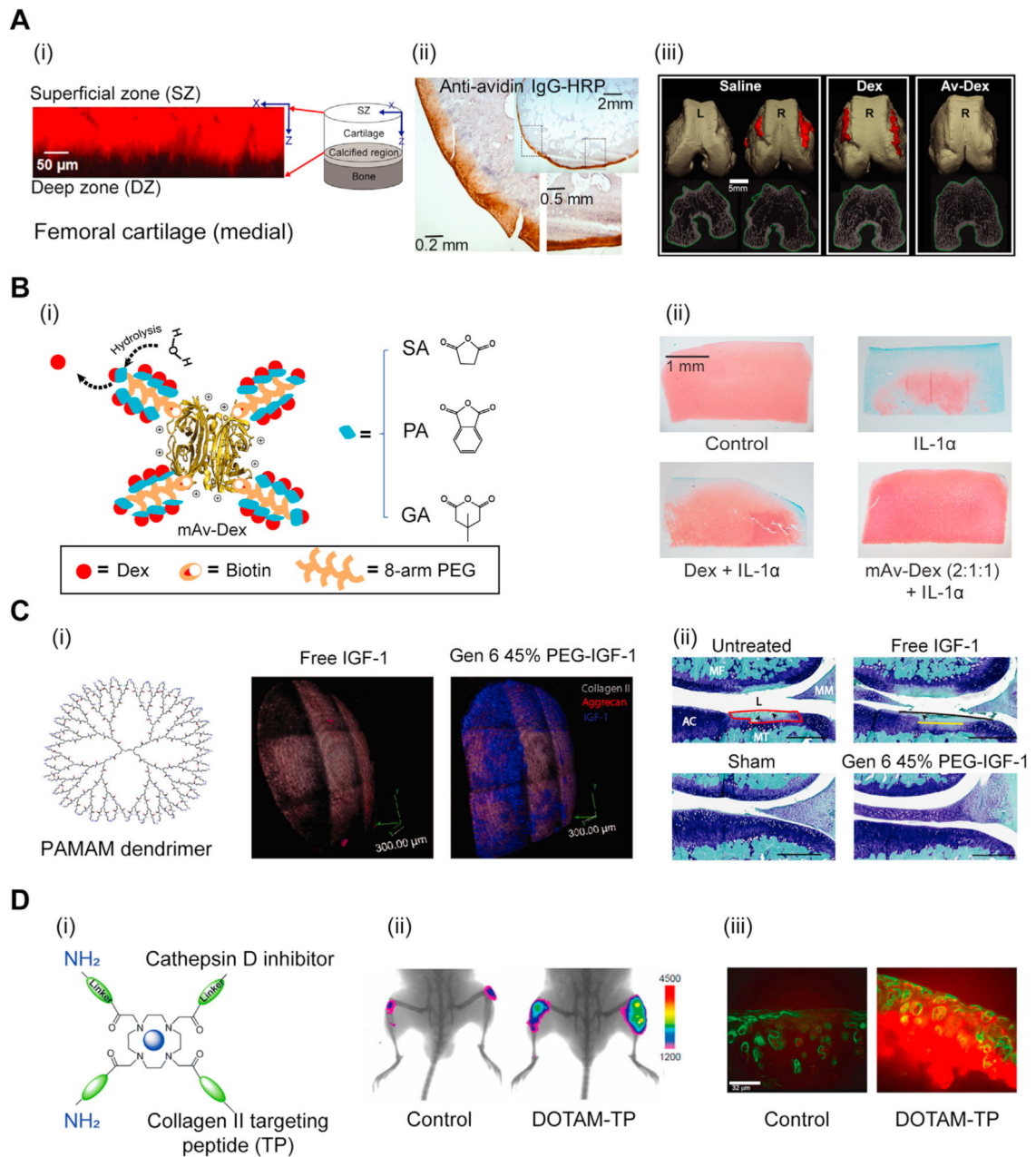
the inner region and decreases towards the outer region. **E.** Intervertebral disc is comprised of gelatinous nucleus pulposus containing a random network of collagen II and proteoglycans.

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**Fig.4.**

A. (i) Full-thickness penetration of positively charged Avidin in rabbit femoral cartilage within 24 h of IA injection. Adapted from [53]. (ii) IHC staining shows presence of Avidin in rabbit cartilage even at 3 weeks after ACLT surgery. (iii) μCT of femoral condyles show significantly suppressed osteophyte volume with Av-Dex compared to free Dex. Adapted from ref [16]. Printed with permission from AO Research Institute Davos. **B.** (i) Multi-arm Avidin nanoconstruct conjugated to Dex (mAv-Dex) using ester linkers. (ii) Safranin-O/fast green staining of cartilage explants shows mAv-Dex significantly suppressed IL-1 induced GAG loss compared to free Dex over 16 days. Adapted from ref [55]. Printed with permission from Elsevier. **C.** Cationic PAMAM dendrimer for IGF-1 delivery. (i) 3D

reconstruction of multiphoton microscopy of rat cartilage. Gen 6 45 % PEG-IGF-1 PAMAM dendrimer shows superior intra-cartilage penetration and retention compared to free IGF-1 at 6 days post IA injection in rats. Aggrecans, collagen and IGF-1 are marked in red, grey and blue, respectively. (ii) Toluidine blue/fast green staining of rat cartilage show PEG-IGF-1 dendrimer suppressed GAG loss greater than free IGF-1 after 4 weeks of ACLT surgery. Adapted from ref [35]. Printed with permission from The American Association for the Advancement of Science. **D.** (i) DOTAM nanoparticles with collagen II targeting peptide (TP) and amines. (ii) Fluorescent images show increased DOTAM-TP retention in mice knees 48 h post IA injection compared to control probe. (iii) Histological analysis of mice cartilage show intra-cartilage localization and retention of DOTAM-TP. Adapted from ref [63]. Printed with permission from American Chemical Society.

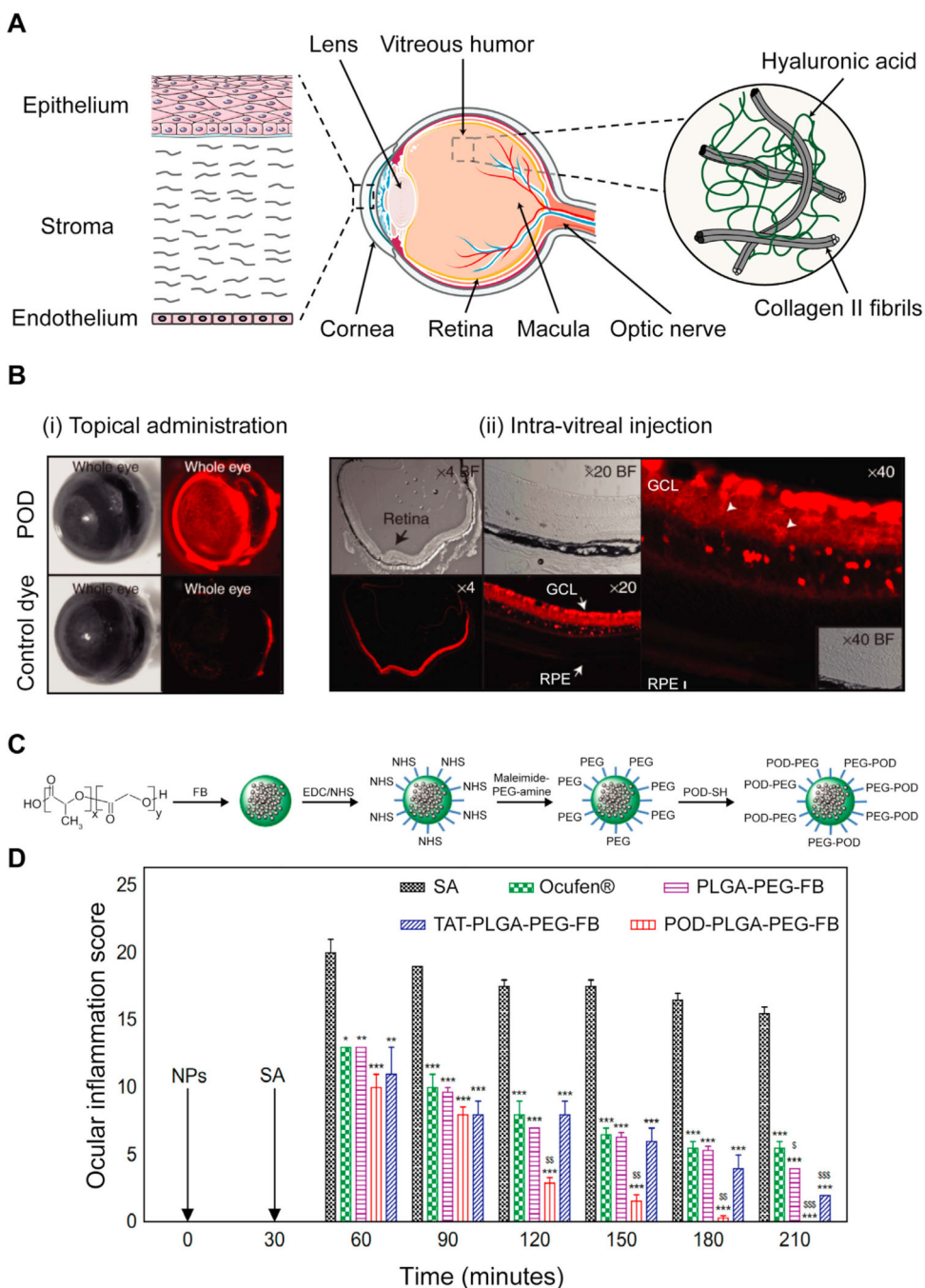


Fig. 5. **A.** Structure of ocular tissue in the anterior (cornea and the lens) and posterior (vitreous humor, retina and macula) regions. Barriers to local drug delivery include negatively charged corneal epithelium layer and vitreous humor comprising of collagen II and hyaluronic acid that prevent diffusion of drugs and their carriers to the posterior regions. **B.** Transport of POD in ocular tissues upon (i) topical administration and (ii) intra-vitreous injection. (i) POD was uptaken by external ocular tissues within 45 minutes upon topical administration in mice eyes while the control free dye only weakly stained the eye. (ii) Intra-vitreous injection

of POD transduced 85 % of neural retina within 2 h. RPE and GCL represent retinal pigment epithelium and ganglion cell layer, respectively. Adapted from ref [137]. Printed with permission from Elsevier. **C.** Synthesis of POD-PLGA-PEG-FB nanoparticles. **D.** Effectiveness of POD-PLGA-PEG-FB in prevention of sodium arachidonate (SA) induced ocular inflammation in rabbit eyes (* $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs inflammation induced by SA. $^{\$}p < 0.05$, $^{\$}p < 0.01$, and $^{\$}p < 0.001$ vs anti-inflammatory effect of Ocufer®). Adapted from ref [140]. Printed with permission under Creative Commons Attribution License.

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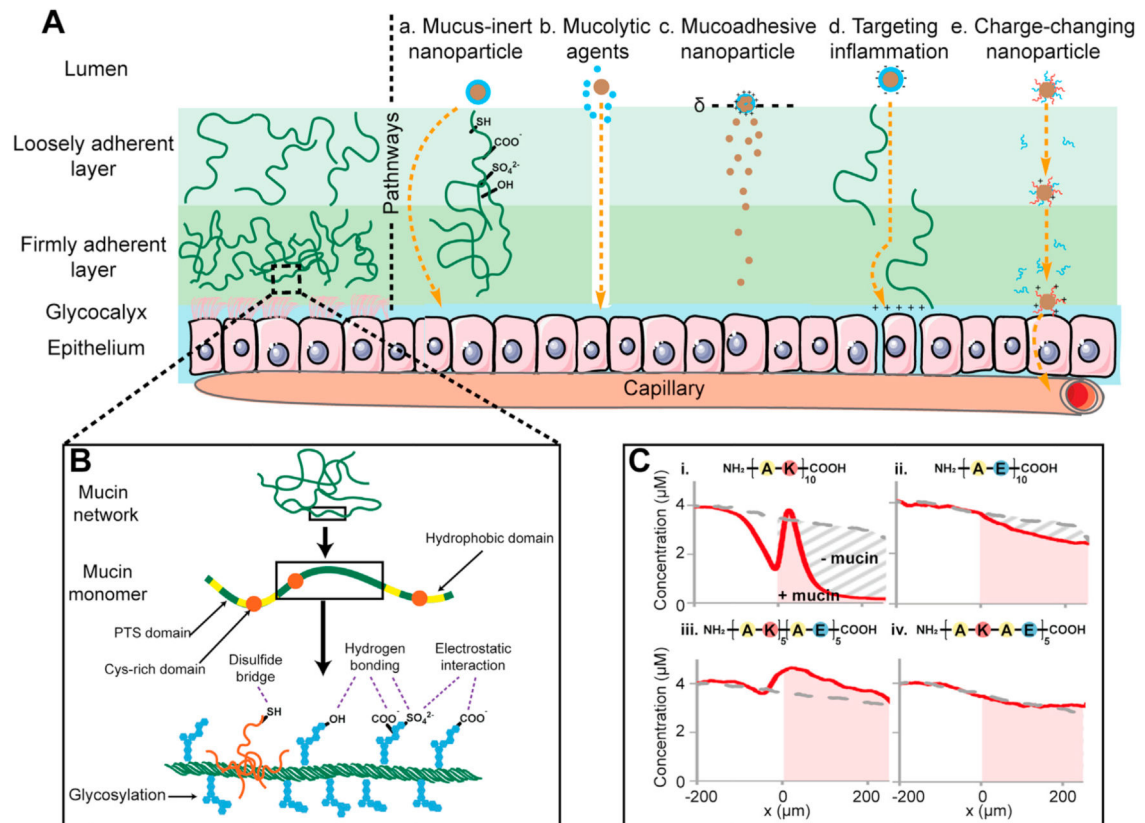


Fig. 6.

A. Characteristics of the mucus barrier coating epithelium and strategies used for promoting mucus penetration (**a.** Mucus-inert nanoparticle **b.** Mucolytic agents **c.** Mucoadhesive nanoparticle **d.** Targeting inflammation **e.** Charge-changing nanoparticle). **B.** Architecture of the mucin network and structure of the heavily glycosylated mucin monomer. **C.** Charge-based transport behavior of short length peptides of varying net charge and spatial distribution in mucin gel, **i.** Cationic peptide, **ii.** Anionic peptide, **iii.** Block peptide and **iv.** Alternate peptide. Adapted from ref [45]. Printed with permission from Elsevier.

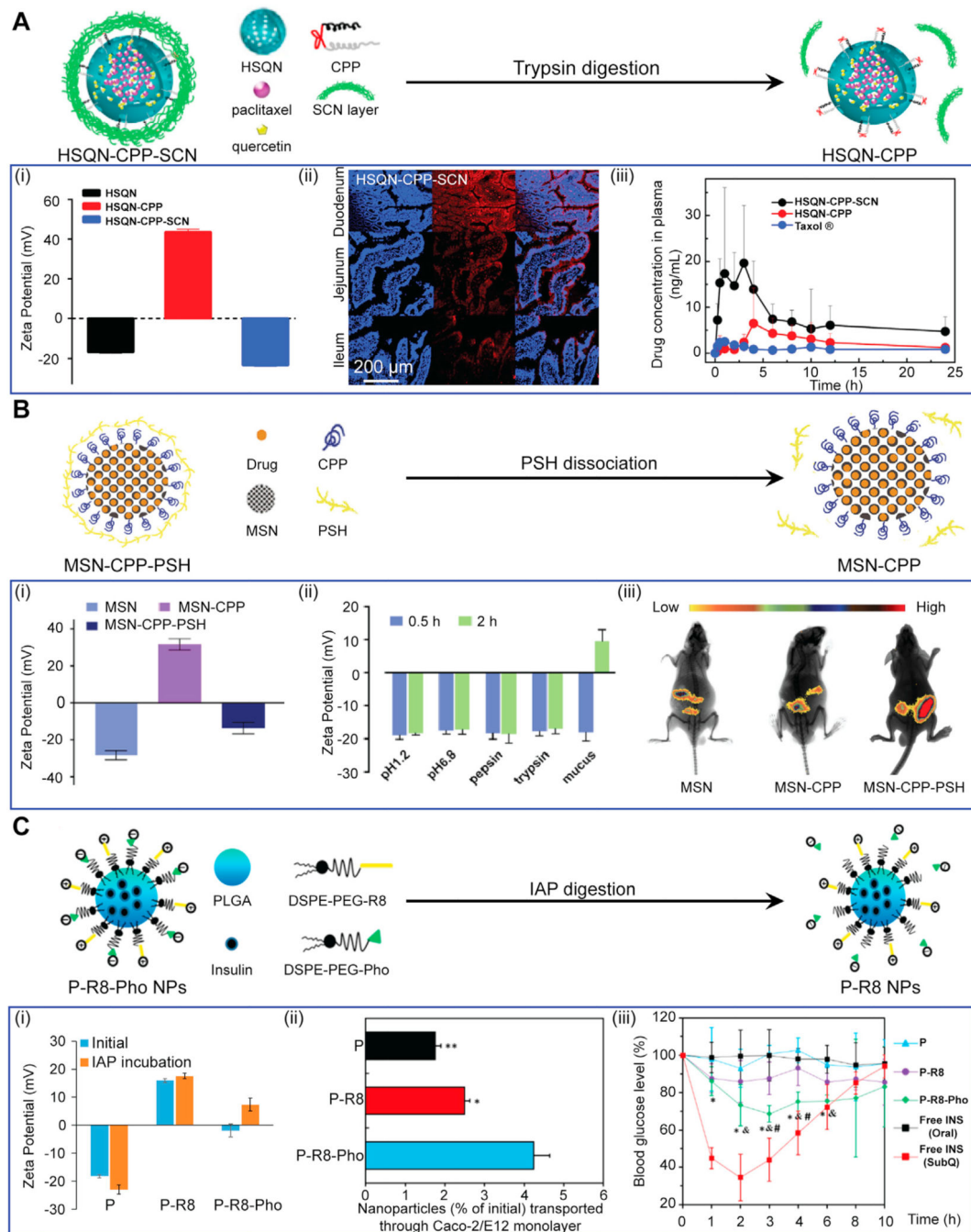
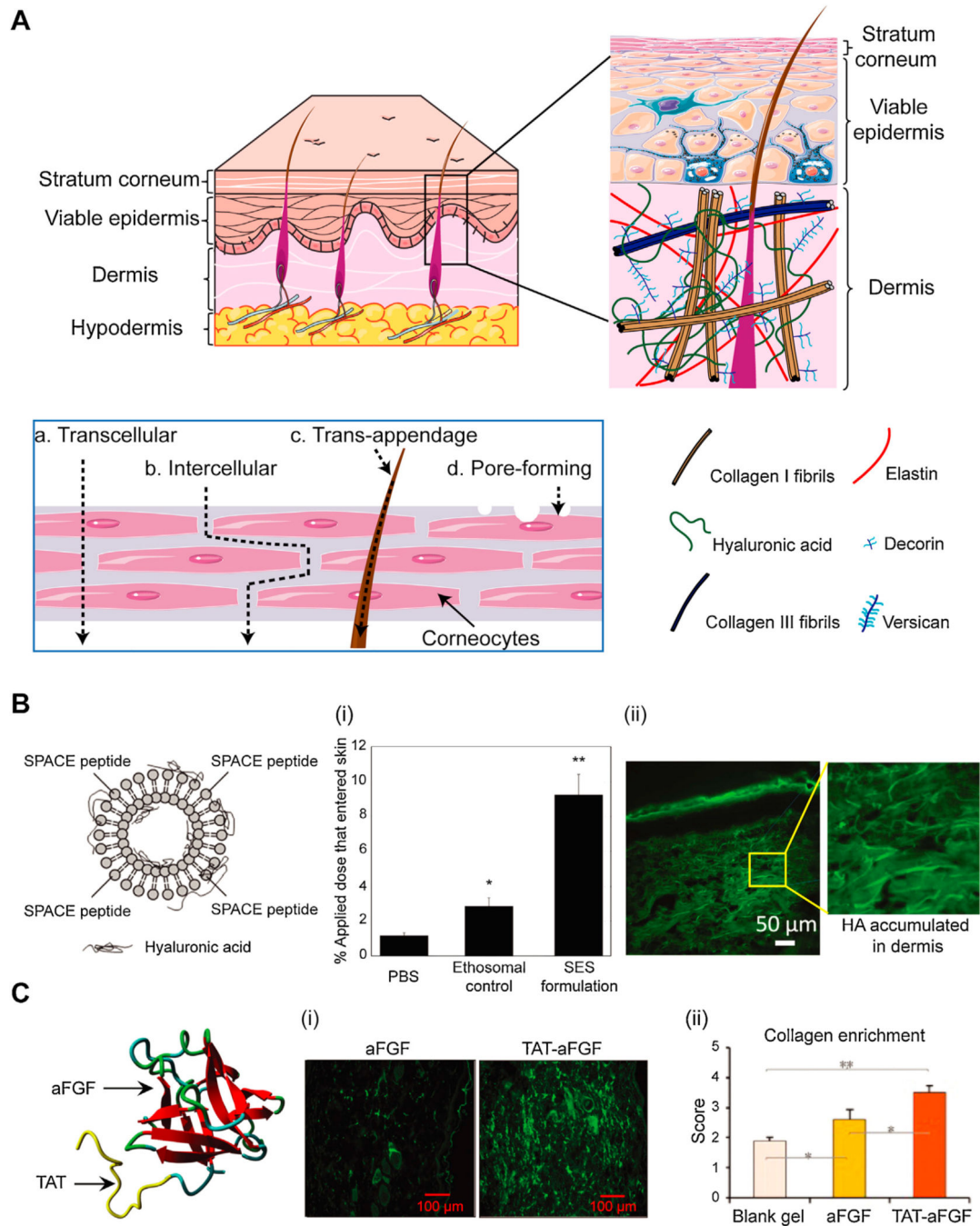


Fig. 7. Charge-reversal nanoparticle systems developed for both effective mucus penetration and GI epithelium targeting. **A.** Quantum dots doped hollow silica nanoparticles (HSQN) modified by CPPs with a cleavable outermost shell of hydrophilic succinylated casein (SCN). (i) Zeta potential, (ii) Confocal laser scanning microscopy images of rat intestine 2 h post oral administration (blue showing cell nuclei and red showing presence of nanoparticles). (iii) Drug levels in plasma after oral administration of HSN-CPP-SCN. Adapted from ref [172]. Printed with permission from Elsevier. **B.** Mesoporous silica nanoparticles (MSN) with an

interlayer of CPP and an outermost shell of thiolated polymer (PSH). (i) Zeta potential, (ii) Enzymatic and pH stability of MSN-CPP-PSH showing specific degradation of PSH shell in presence of mucus that reverses its net charge. (iii) In vivo bio-distribution of MSN-CPP-PSH after oral administration in mice. Adapted from ref [173]. Printed with permission from Elsevier. C. PLGA nanoparticles coated with R8 and anionic phosphoserine (Pho), which dissociates upon digestion with intestinal alkaline phosphatase (IAP) leading to a net positive surface charge. (i) Zeta potential, (ii) Enhanced in-vitro transepithelial transport of P-R8-Pho (iii) Suppression of blood glucose levels after oral administration of insulin-loaded P-R8-Phonanoparticles in diabetic rats. Adapted from ref [174]. Printed with permission from American Chemical Society.

**Fig. 8.**

A. Different layers of skin (stratum corneum, viable epidermis, dermis and hypodermis). Sublayers of viable epidermis and dermis are comprised of a collagen network, elastin, versican and HA. Different routes of transdermal drug delivery (**a.** Transcellular, **b.** Intercellular, **c.** Trans-appendage and **d.** Pore-forming). **B.** SPACE peptide conjugated to phospholipids designed as an ethosomal carrier system (SES) to deliver hyaluronic acid (HA). (i) Percent penetration of topically applied dose into total skin. (ii) Confocal image of dermis showing SES can penetrate through the full skin to deliver HA. Adapted from

ref[196]. Printed with permission from Elsevier. **C.** Conjugation of TAT to aFGF to improve its transdermal delivery efficiency. (i) Immunofluorescence of aFGF or TAT-aFGF accumulated in dermal and subcutaneous tissues. (ii) Semi-quantitative scoring showing greater collagen enrichment in rat skin injury model with TAT-aFGF. Adapted from ref[199]. Printed with permission under Creative Commons Attribution License.

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Table 1

GAG content of various negatively charged tissues is represented as $\mu\text{g GAG/mg dry tissue weight}$. Average fixed charge density (FCD) of tissues is calculated using $\frac{\text{GAG content } (\mu\text{g})}{\text{MW}_{\text{GAG}} \cdot \text{Tissue water } (\mu\text{l})} \times \frac{\text{net charge}}{\text{mol}}$. For sulfated GAGs, MW \sim 458 g/mol and -2 moles of charge per mole of GAG disaccharide unit is assumed [236]. For HA, MW (of repeating disaccharide unit) \sim 401 g/mol and -1 mole of charge per mole of repeating unit is assumed. Tissue dry weight was converted to wet weight by using their respective water content. Cartilage, ligament/tendon, meniscus, nucleus pulposus, vitreous humor, mucus and skin water content were assumed 80 %, 80 %, 70 %, 80 %, 99 %, 95 % and 70 %, respectively. Additionally, percentage composition of different types of GAGs (chondroitin sulfate (CS), keratan sulfate (KS), heparan sulfate (HS), dermatan sulfate (DS) and hyaluronic acid (HA)) in each tissue is presented.

| Tissue | GAG content ($\mu\text{g GAG/mg dry tissue}$) | Average Fixed Charge Density (mM) | Types of GAGs |
|--------------------------|---|---|--|
| Cartilage | 100 to 300 [221,222] | -170.0 [223] | CS (80 %) KS (5-20 %) HA (1-10%) [49] |
| Synovial fluid | 28.8 [224] | -8.8 [224] | HA (97%) CS (3 %) [224] |
| Ligaments and tendons | 2 to 35 depending on region [91] | Ligament: -17.9 [82] Tendon: -6.6 [82] | CS (16-56 %) DS (20 %-66 %) [225] |
| Meniscus | 39 to 9 from inner to outer region [226] | -51.5 [53] | CS (60 %) DS (20 %) KS (15 %) HA (3 %) [227] |
| Nucleus pulposus | 100 to 400 [228] | -138.2 [229] | CS (67 %) KS (21 %) HA (5%) [230] |
| Vitreous humor | 23 [231] | -0.6 [231,232] | HA (96.2 %) CS (3.5 %) HS (0.3%) [231] |
| Gastric mucosal membrane | 19.6-23.4 [233] | -4.4 [233] | DS (45 %) CS (20 %) HS (19 %) HA (16 %) [234] |
| Skin | 3.4 [235] | -2.5 [235] | DS (46.7 %) HA (46 %) HS (3.9 %) CS (3.3 %) [235] |

Table 2

Transport studies using peptides and proteins with different net charges (z) across tissues of varying FCD.

| Peptide/Protein | Sequence | z | Cargo | Tissue | Ref. |
|------------------------------|-----------------------------|---------|--|---------------------|----------------------------------|
| TAT/TAT derivative | GRKKRRQRRRPQ or RKKRRORRRGC | +8 | β -galactosidase, Endostatin, aFGF, μ -calpain inhibitory peptide, c-Jun N-Terminal kinase inhibitor | Ocular | [42,125,126,131,134,135,136,142] |
| Arginine-rich peptides | CGGGYGRKKRRQRRR | +8 | Insulin | Skin, Hair follicle | [199,200,201,210] |
| | (RRAAA) ₃ RR | +8 | - | Caco-2 monolayer | [40] |
| | RRRR(AARRR) ₃ R | +14 | - | Cartilage | [14] |
| | R ₈ GC | +8 | - | Cartilage | [61] |
| | R ₈ K | +9 | - | Ocular | [131] |
| | R ₆ | +6 | Bevacizumab, Ranibizumab | Ocular | [141] |
| | (D)-R ₈ | +8 | Insulin | Mucus | [161] |
| | CR ₈ | +8 | Insulin | Mucus | [174] |
| | R ₇ | +7 | Cyclosporine A | Skin | [198] |
| | WYRGRL | +2 | Dexamethasone, Cathepsin D inhibitor | Cartilage | [20,62,63] |
| Collagen II-binding peptide | KKKRKGKGLGKKRDPCLKKYK | +11 | Insulin like growth factor 1 | Cartilage | [58,59,60] |
| | - | +6-20 | Dexamethasone | Cartilage | [15,16,53,54,55,82] |
| Avidin | DWRVHPPRPSAC | +1 | siRNA | Cartilage | [66] |
| Chondrocyte affinity peptide | VLTTGLPALISWIRRRHRRHC | +7 | siRNA | Cartilage | [68,70] |
| p5RHH | KETWETWTEWSQPKKRRKV | +3 | GRX-1 | Cartilage | [71] |
| PEP-1 | PIRRRKKLRRLK | +8 | FK506 binding proteins | Ocular | [127,128] |
| PTD-4 | RRQRRTSKLMKR | +7 | - | Synovium | [83] |
| PTD-5 | KRIHQRLSRNS | +4 | - | Synovium | [83] |
| PTD-2 | SFHQFARATLAS | +2 | NF- κ B-blocking peptide | Synovium | [84,85,86] |
| HAP-1 | CKSTHRLC | +2 | Interleukin-4 | Synovium | [89] |
| SyETP | RQIKWVFQNRMRMKWKKK | +8 | Red fluorescent protein plasmid, Antisense oligonucleotide | Ocular | [123,124,131,132] |
| Penetratin | GGG(ARKKAAKA) ₄ | +16 | siRNA, plasmid DNA, Flurbiprofen | Ocular | [137,138,139,140] |
| Peptide for ocular delivery | AKAKAKAKAKAEAEAEAE | Neutral | - | Mucus | [45] |
| Lysine-rich peptides | | | | | |

| Peptide/Protein | Sequence | z | Cargo | Tissue | Ref. |
|--------------------------|-----------------------------|---------|---------------------------------|-----------|-----------|
| | AKAEAKAEAKAEAKAEAKAE | Neutral | - | Mucus | [45] |
| | (AK) ₇ ANANAN | +7 | - | Cartilage | [14] |
| | (AK) ₇ AFAFAF | +7 | - | Cartilage | [14] |
| Cell penetrating peptide | CRQKIWFQNRMRMKWKK | +7 | Paclitaxel, Lopinavir | Mucus | [172,173] |
| Cyclopeptide TD-34 | ACSSKSKKHCG | +4 | Insulin, Cyclosporine A | Skin | [195,198] |
| SPACE | ACTGSTQHQQG | +1 | Hyaluronic acid, Cyclosporine A | Skin | [196,198] |
| T2 | LVGVFH | +1 | 5-fluorouracil | Skin | [209] |
| IMT-P8 | RRWRWRNRFNRRRCR | +9 | Pro-apoptotic peptide | Skin | [210] |
| Magamin | GIGKFLHSAKKFGKAFVGEIMNS | +4 | - | Skin | [211] |
| Transportan | GWTLNSAGYLLGKINLKALAALAKKIL | +4 | Paclitaxel | Skin | [216] |