Development of Polymeric Carriers for the Treatment of Acute Kidney Injury

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Development of Polymeric Carriers for the Treatment of Acute Kidney Injury

by

Yi Chen

A DISSERTATION

Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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Under the Supervision of Professor David Oupický

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Abstract:

Development of Polymeric Carriers for the Treatment of Acute Kidney Injury

Yi Chen, Ph.D.

University of Nebraska Medical Center, 2018

Supervisor: David Oupický, Ph.D.

Acute kidney injury (AKI) is a major kidney disease associated with high mortality and morbidity. Long-term AKI may lead to chronic kidney disease and end-stage renal disease. Several clinical trials failed due to lack of efficacy and undesired side effects. Studies showed that macromolecular delivery systems would be a promising method to target kidney, however, little is known about how physicochemical properties affects the polymers deposition in ischemia-reperfusion (I/R) AKI.

Gene therapy has been well studied as a promising therapeutic agent for several diseases, including cancer and AKI. Although small interfering RNA (siRNA) has been commonly used to treat AKI through hydrodynamic injection, this method has some disadvantages.

The expression of CXCR4 increases in response to AKI. Emerging evidence shows that CXCR4/SDF-1 axis is implicated in regulating trafficking and invasion of inflammatory cells in the injured kidneys. The inhibition of the axis appears to exert beneficial therapeutic effect in AKI.

First, to understand how physicochemical properties affects renal accumulation in AKI, we synthesized a panel of 9 fluorescently labeled polymers with a range of size and
different net charge. By testing biodistribution in unilateral I/R animal model, we found negatively charged pMAA-5 and neutral pHMA-36 had greatest potential for accumulating in I/R kidneys as compared with sham-operated kidneys. The polymers passed through glomerulus and sustained in proximal tubular cells for up to 24 hours after injection. We also confirmed the consistency of bilateral and unilateral I/R animal model by confirming biodistribution of pAPMA-30 and pHMA-16 in bilateral I/R animal model. This study demonstrated for the first time that polymers with specific physical characteristics exhibit promising enhanced ability to accumulate in AKI kidney.

Second, to explore the potential of CXCR4/SDF-1 axis in the treatment of AKI, we formulated polyplexes with our previously synthesized polymeric CXCR4 antagonist (PCX). Biodistribution study indicated that the majority of injected polyplexes can accumulated in injured renal rubule cells. Transfection effect of the polyplexes in unilateral I/R injury mouse model showed a better silencing effect compared with hydrodynamic injection, which providing a novel dual-functional pharmacological method for treatment of AKI.
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List of Abbreviations

AKI – acute kidney injury

CXCR4 – C-X-C chemokine receptor type 4

PCX – polymeric CXCR4 antagonist

SDF-1 – stromal cell-derived factor 1

GBM – glomerular basement membrane

DLS – dynamic light scattering

RNAi – RNA interference

siRNA – small interfering RNAs

ATP – adenosine triphosphate

NO – nitric oxide

ROS – reactive oxygen species

NADPH – nicotinamide adenine dinucleotide phosphate

DLS – dynamic light scattering

Scr – serum creatinine

BUN – blood urea nitrogen

LMWC – low molecular weight chitosan

TEM – transmission electron microscopy
RES – reticulo-endothelial systems

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

I/R – ischemia-reperfusion
Chapter 1. Introduction

1. Renal Anatomy and Selective Permeability

1.1. Renal Anatomy

Kidneys are organs that are highly vascularized and function as osmoregulators by selective filtration, resorption, secretion and excretion. As shown in Scheme 1A, one kidney of healthy adult human contains about one million nephrons which are the units of kidney [1]. One nephron is composed of a renal corpuscle and a hairpin-shaped tubule. Renal corpuscle consists of a cluster of capillaries named glomerulus and Bowman’s capsule. Proximal tubule, loop of Henle, distal tubule and collecting duct make up to the whole tubule system. The glomerulus prevents large molecules, blood cells and most proteins from elimination while filtering fluid and waste products into tubules in which several minerals are reabsorbed back to bloodstream and wastes are removed into urine.

1.2. Selective Permeability

The glomerular filtration barrier, located within the glomerulus of the nephron, is made up of three different parts, including glomerular endothelial fenestrations [2], glomerular basement membrane (GBM) [3], and podocytes [4] (Scheme 1B). The glomerular endothelial fenestrations have the pore size of around 100 nm in diameter [5]. The GBM is a 300-nm-thick connective tissue membrane with presence of heparin sulfate [6] and negatively charged proteoglycans. Its average pore size is 3 nm [7]. The GBM serves as a size and charge-selective filtration barrier. The podocyte slit pores attached over the surface of the GBM towards to the urinary space has average width of 15 nm [8].
Taken together, the glomerular filtration barrier functions as an effective size cutoff for the molecules or particles with size less than 10 nm to enter urinary space from the blood circulation [9]. In other words, structural features of the glomerular barrier can be a criterion for nanoparticles design to target kidney in diverse renal diseases.

The synthesis and application of nanoparticles have been attracted much attention in biopharmaceutical areas, such as drug delivery [10, 11], therapeutics [12] and diagnostic imaging [13, 14]. Effective therapeutic outcomes require specific features of nanoparticles to overcome biological barriers, accumulate in target tissue and escape from rapid clearance [15, 16]. Therefore, it is of great importance to understand the effect of different properties of nanoparticles on biological distribution [17].
Scheme 1. Renal anatomy. A) Nephron of kidney which consists of renal corpuscle and hairpin-shaped tubules. B) Detailed structure of glomerulus (Adapted from [18]).
1.2.1 Nanoparticle Size

The effect of nanoparticle size on *in vivo* biodistribution has been widely investigated by several studies. Nanoparticles smaller than 10 nm can be subject to rapid clearance by kidney and be excreted from the body. Over 50% of injected dose of nanoparticles can be excreted via renal clearance 4 h after administration if hydrodynamic diameter is below 5.5 nm [9, 15]. Larger nanoparticles over 100 nm are taken up and degraded by spleen and liver [19]. Nanoparticles with size between these two ranges (10-100 nm) typically have longer circulation time in blood and will not be filtered by kidney into the urine unless they are degraded into smaller particles [20, 21]. Given that some studies have shown inorganic nanoparticles with the size ranging from 12-16 nm passed through this filtration threshold [22], this may not be a strict rule for renal clearance of all types of nanoparticles and each study should be case-by-case due to distinct chemical composition and size-distribution profile of each type of nanoparticles.

1.2.2 Nanoparticle Charge

The surface charge of nanoparticles plays an important role in determining nonspecific cellular internalization and protein absorption during circulation which are the concerns when it comes to systemic delivery. Among nanoparticles with positive, negative and neutral charge, highly positively charged nanoparticles have the highest rate of cell uptake. It has been also well accepted that nanoparticles with highly positive charge are more subject to protein binding and uptake by phagocytes, leading to high nonspecific internalization rate and rapid clearance by reticuloendothelial system (RES) [23, 24]. Compared to neutral nanoparticles, nanoparticles with negative charge still have more
non-specific cellular uptake [25]. Yamamoto et.al. demonstrated negatively charged micelles exhibited less uptake in liver and spleen than neutral micelles due to synergic steric and electrostatic repulsion [26].

Given the negative charge of the GBM, charge selectivity is also an important criterion of nanoparticles design for kidney filtration or targeting different renal cells. Gold nanoparticles with positive charge accumulate in glomeruli 24 h after administration, whereas neutral and negatively charged gold nanoparticles do not. Neutral gold nanoparticles accumulate extensively in the arteries and negative gold nanoparticles are distributed in the kidney homogeneously, indicating the influence of surface charge on gold nanoparticles filtration by kidney [27].

1.2.3 Nanoparticle Shape

The structural features of nanoparticles are also responsible for dramatically different renal clearance profile which is important aspect for design of targeted drug delivery systems. Although it is well recognized that nanoparticles less than the 10-nm cutoff of the kidney filtration barrier can pass through GBM, nanoparticles larger than 10 nm but with high aspect ratios have been demonstrated to cross GBM paradoxically. Several studies have demonstrated that single-walled carbon nanotubes with average diameter less than 10 nm and average size 100 to 500 nm had rapid and effective renal clearance with half-life of a few hours [28-31]. Based on the multimodal imaging methods, including simultaneous dynamic positron emission tomography, near-infrared fluorescence imaging and microscopy, Ruggiero and colleagues demonstrated that hydrostatic forces
in glomerulus make the long axis of carbon nanotubes in a perpendicular orientation to GBM, thereby their short axis is oriented horizontally to GBM [30]. Another group showed the consistent result by transmission electron microscopy imaging [31].

2. Acute Kidney Injury

Acute kidney injury (AKI) can be characterized by an abrupt decline in the glomerular filtration rate over a period of minutes to days that inhibits balance of electrolyte, fluid and acid/base [32]. Clinically, AKI is mainly triggered by toxic responses to medications, ischemia which results from a reduction of blood flow in kidney by decreased cardiac output (cardiac surgery), and sepsis [33-35]. It is a complex disorder and impacts about 13.3 million people annually [36]. There are about 1.7 million people who die annually from AKI in the world [37-39]. The long-term outcomes of AKI for patients may further develop into chronic kidney disease and end-stage renal disease, which can lead to poor life quality and high long-term costs [40, 41]. Although people have achieved substantial progress in treating AKI clinically by intensive care medicine and dialysis, the prognosis has not significantly been improved and the mortality remained 30-50% over the last decades [42]. Therefore, AKI is a major global health problem that is associated with high morbidity and high mortality.

In the past, there has been no uniform accepted definition for AKI, which made it difficult to clarify the incidence of AKI and hindered early recognition and management of AKI. As lots of efforts of basic and clinical researchers have been focused on AKI over the last decades, there is a great need to define a uniform criteria for AKI. The Kidney
Disease: Improving Global Outcomes (KDIGO) group has recognized these limitations and proposed a universal definition and staging system of AKI for practice, research and public health [43], which describes that serum creatinine and urine output are the standard diagnostic biomarkers for measuring kidney function. As is shown in Table 1, AKI occurs within a broad spectrum ranging from the small changes in blood chemistry of patients (mild injury) to renal failure that needs renal replacement therapy (most severe impairment).
Table 1. KDIGO staging system for AKI (Adapted from [43]).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Serum creatinine</th>
<th>Urine output</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5–1.9 times baseline OR ≥0.3 mg/dl (≥26.5 μmol/l) increase</td>
<td>&lt;0.5 ml/kg/h for 6–12 hours</td>
</tr>
<tr>
<td>2</td>
<td>2.0–2.9 times baseline</td>
<td>&lt;0.5 ml/kg/h for ≥12 hours</td>
</tr>
<tr>
<td>3</td>
<td>3.0 times baseline OR Increase in serum creatinine to ≥4.0 mg/dl (≥353.6 μmol/l) OR Initiation of renal replacement therapy OR In patients &lt;18 years, decrease in eGFR to &lt;35 ml/min per 1.73 m²</td>
<td>&lt;0.3 ml/kg/h for ≥24 hours OR Anuria for ≥12 hours</td>
</tr>
</tbody>
</table>
2.1 Pathophysiology of AKI

The mechanisms of AKI have been well studied by researchers in recent decades. The understandings most derived from the work in animal models have provided promising insight into the underlying pathophysiology [44, 45]. Based on animal models of AKI that represent renal ischemia-reperfusion injury and nephrotoxicity, AKI is most likely to be a complicated disease that can be induced by several potential pathways and mechanisms of injury. Generally, the coagulation system is locally activated when the kidney has energy failure or renal cells are exposed to toxic medications [35, 46]. Leucocytes infiltrate the kidney [47], inflammatory cytokines and chemokines are released [48-50] and oxidative stress is activated [51]. The activation of coagulation, oxidative stress and inflammatory infiltration will further lead to vascular endothelial cell damage and microvascular congestion [52-54]. The sustained activation of these pathogenic factors will again amplify the inflammatory cascades, leading to apoptotic cell death [55, 56]. Some changes also occur on intracellular level such as loss of polarity [57] and adhesion to the basement membrane tubular cells [58]. Moreover, AKI induces distant organ dysfunction called organ cross-talk including liver, heart, lung and brain, emphasizing the complexity of its biological response [59, 60].

2.1.1 Microvasculature Injury

The renal microvasculature plays a key role in determining vascular tone, leukocyte function in the pathophysiology of AKI [61]. Kidney is an organ with a high energy demand but a relatively low net oxygen extraction, which makes it susceptible to the changes of renal oxygen during any vascular ischemia and perfusion [48]. In healthy state,
adequate oxygen supply to the kidney is necessary for homeostatic control of renal function by the production of mitochondrial adenosine triphosphate (ATP), nitric oxide (NO) and reactive oxygen species (ROS) [62]. Once the kidney is injured, microvasculature system is damaged and the balance between ATP, NO and ROS is broken, leading to a series of subsequent pathogenic effects such as hypoxia and oxidative stress. The endothelial cells are activated and express new cell surface markers due to damage of microvascular endothelium and changes in the glycocalyx, which further promote the recruitment and adhesion of leukocytes and platelets, change oxygen delivery and trigger additional inflammation and endothelial cell injury [63, 64]. In addition, oxygen delivery and supply can be influenced by increase in vascular permeability, and production of vasoconstrictive prostaglandins and oxidative stress by injured tubule cells [63, 64]. The reduction in vascular endothelial growth factor (VEGF) and increase in transforming growth factor beta (TGF-β) signaling pathways would result in reduced density of peritubular capillary that is responsible for the development of hypoxia and renal fibrosis [65].

### 2.1.2 Inflammation

Immune response can be classified into two different types: innate immune system and adaptive immune system, both of which are essential for the initiation of pathophysiology of AKI. The innate immune system has cells that are already present in the body and responsible for fighting microbes at the early response to injury. Such cells include macrophages, neutrophils, dendritic cells (DCs), natural killer (NK) cells and natural killer T cells. Besides, soluble molecules and membrane-associated receptors such
as complement, cytokines and toll-like receptor (TLR) are also important contributors to tubular injury. On the other hand, the adaptive immune system is initiated by specific pathogens that are able to overcome innate immune defenses. It comprises DC maturation and antigen presentation, T lymphocyte activation and proliferation, as well as T to B lymphocyte interactions, which are normally silent.

It is well known that robust inflammation response is activated following renal ischemia reperfusion [66]. Such activation triggered by the damage of tubule cells includes not only innate immune response but also adaptive immune response. During reperfusion, blood rich in innate immune components passes through injured kidney and accelerates the renal damage. A number of pro-inflammatory cytokines and chemotactic cytokines, such as TNF-α, IL-1β, IL-6, TGF-β and ENA-78 (epithelial neutrophil-activating protein 78), are synthesized and produced following renal ischemia reperfusion injury [66]. Cytokines have been proved to be responsible for both local and distant organ injury in AKI. Renal injury is attenuated during ischemic acute renal injury by means of direct blockade of certain cytokines [67, 68]. Additionally, leukocytes are recruited into the post-ischemic kidney, leading to increase in vascular permeability and destruction of epithelial and endothelial cell integrity [69]. Meanwhile, the tubular injury also activates DCs and thus activates naïve T cells to proliferate in an antigen-specific fashion, implicating the initiation of adaptive immune response.

2.1.3 Reactive Oxygen Species
Kidney is an organ that can not only directly produce a small amount of ROS in the course of renal oxidative metabolism [70], but also is susceptible to the damage by ROS [71-73]. Healthy kidney tolerates ROS without any obvious adverse effects only if their concentration is maintained at a low level. However, excessive ROS produced by renal cells themselves or infiltrating cells (neutrophils and macrophages) in injured kidney can contribute to, or even further exacerbate several types of renal cell damage [71, 74]. During ischemia, the reduction of molecular oxygen within cells leads to intracellular and extracellular ATP depletion, producing increased ATP degradation products including adenosine, hypoxanthine, xanthine and a shift towards anaerobic metabolism [75]. However, on reperfusion, the initial increase in oxygen delivery amount exceeds the amount needed for cellular metabolism from anaerobic pathways back to aerobic pathways. The whole process would lead to generation of damaging free radicals and a wide variety of ROS are generated, including superoxide radical, hydrogen peroxide and hydroxyl radical.

ROS contribute to cellular signaling and further affect multiple aspects of cellular functions such as proliferation, migration, gene expression and apoptosis [76]. Generally, there are thought to be three main pathways for the generation of ROS during ischemia-reperfusion injury: 1) conversion of xanthine dehydrogenase to xanthine oxidase, 2) nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and 3) uncoupling of the mitochondrial electron transport chain. Several studies have demonstrated that treatment with antioxidants or free radical scavengers can diminish acute renal injury. Noiri et al. showed the protection effect on rat kidney against ischemia
by targeting inducible nitric oxide synthase with oligodeoxynucleotides [77]. Ma and colleagues reduced cisplatin-induced AKI using saikosaponin-D which acts as a ROS scavenger by inhibiting MAPK and NF-κB signaling pathways [78].

### 2.1.4 Apoptosis

Apoptosis, an energy-dependent and programmed cell death, is another major etiological factor of AKI, which occurs in epithelial cells when they are undergoing a severe renal injury. As shown in Scheme 2, the mechanisms of apoptosis are complex and can be divided into two major pathways during ischemic renal tubule cell injury: intrinsic and extrinsic pathways.

It is now widely accepted that mitochondria is a major site for apoptosis and plays an important role in determining and regulating intrinsic and extrinsic pro- and anti-apoptotic process [79, 80]. During ischemic renal injury, stress-induced increase of mitochondrial permeability permits the production of free radicals [79], ATP depletion [80] and release of pro-apoptotic proteins, cytochrome c, and in turn activates caspase-9 [81]. The mitochondrial permeability transition has been well recognized to be regulated by apoptotic effectors, Bcl-2 family proteins [82]. Bax and Bak are the pro-apoptotic members of Bcl-2 family proteins that increase mitochondrial membrane permeability and promote apoptosis, whereas Bcl-2 and Bcl-XL are anti-apoptotic factors that maintain mitochondrial integrity and thus suppress apoptosis. During renal ischemia-reperfusion injury, the Bax/Bcl2 ratio, a decisive factor for programmed cell death [83], is increased due to the activation of Bax [84, 85] and inactivation of Bcl2 [86, 87]. Inhibition of apoptosis by blocking caspase or Bcl-2 proteins in ischemia animal models motivated the
development of therapeutic treatments [88, 89]. Mei et al. made double knockout of Bax and Bak in mice kidney proximal tubules and found ameliorated apoptosis in unilateral urethral obstruction model. This finding indicated that Bax and Bak together play a key role in mediating intrinsic apoptosis in AKI [90].

Extrinsic apoptosis pathway is induced by the binding of death receptors and death ligands of the tumor necrosis factor (TNF) family on the cell surface. Death receptors include Fas and tumor necrosis factor-alpha (TNF-α). Activation of such death receptors leads to activation of downstream caspases to induce apoptosis, such as caspase 8 and caspase 10 [91].
Scheme 2. Major intrinsic and extrinsic signal pathways of apoptosis in renal cells exposed to stress (Adapted from [56]).
2.2 Diagnosis and Management of AKI

AKI clinical evaluation mostly includes two parts: history examination and thorough physical examination. These two examinations are critical for determining and categorizing AKI. The history examination should be divided into drug history and social history examinations. Drug history should be considered for any uses of over-the-counter formulations, herbal remedies, and recreational drugs. Any exposure to tropical disease should be taken as social history. For physical examination, fluid status of patients should be monitored, any signs of acute and chronic heart failure, as well as infections should be examined, any skin rashes can also indicate systemic illness. Laboratory evaluation should include parameters such as serum creatinine (Scr), blood urine nitrogen (BUN), electrolytes, complete blood count and differential urine output.

Due to the complexity of AKI, all medications that underwent clinical trial failed and there are no effective pharmacotherapies for treating AKI. So far the clinical management of AKI is still primarily supportive. The key to management is to correct hypovolemia and assure adequate renal perfusion through fluids resuscitation [92]. PBS or human albumin fluid are preferred for fluid resuscitation over hydroxyethylstarch because of its nephrotoxicity [93]. In addition, control of electrolyte imbalance is essential for prevention or treatment of AKI. Insulin and calcium gluconate and sodium polystyrene sulfonate (Kayexalate) are common methods that can be used to control electrolyte imbalances, such as hyperkalemia, hypermagnesemia, hyponatremia, hypernatremia and metabolic acidosis. Besides these treatments, some other auxiliary therapies should be involved simultaneously with standard management practices.
When AKI has potentially life-threatening complications, such as uncontrolled symptomatic fluid overload, severe persistent metabolic disturbances and refractory hyperkalemia, renal replacement therapy is required [94, 95]. However, the timing of initiating renal replacement therapy is still debatable [96, 97] and the cost of dialysis is a major burden for patients and healthcare systems. Based on recent advances in renal injury and repair signaling pathways, several pharmacological agents including antioxidants, anti-apoptosis or anti-inflammatory agents, growth factors and vasodilators for improving renal functions have developed [98]. However, so far no pharmaceutical agents are successful in clinical trials [99-101].

3. Animal Models of AKI

To better understand pathophysiological mechanisms or study pharmacological therapies of AKI, in vivo animal models are indispensable to mimic situation in human body [102]. There are various animal models of AKI have been developed and tested. The most commonly used animal models for AKI are renal ischemia-reperfusion (IR) model and nephrotoxicity model which is induced by drugs like cisplatin. The IR model can be divided into unilateral and bilateral renal IR model on the basis of the site of injury [103-105]. The unilateral renal IR model can further be categorized as two subtypes: unilateral IR with contralateral nephrectomy [105, 106] or without contralateral nephrectomy [107, 108]. Compared to unilateral IR model, the bilateral IR model is more common because it is better to mimic the condition of human pathology [109, 110]. However, compared with nephrotoxicity model, these models might have big variations leading to inconsistency in results.
4. **Kidney-targeted Drug Delivery Systems**

Nanoparticles have attracted increasing attention over the past few decades due to their effective diagnostic and therapeutic functions in several diseases, such as cancer. Nanoparticles can be engineered to improve delivery efficacy to focus by overcoming several barriers that are associated with systemic drug administration, thus minimize non-specific accumulation and side effect in other organs. Lots of carrier systems have been developed and employed to optimize nanoparticles for prolonged circulation time in blood, targeting to specific organs or cell types. [111-113]. For example, Yang and colleagues developed chitosan/siRNA nanoparticles that are not only taken up specifically by renal proximal tubule epithelial cells through megalin-mediated pathway, but also enable gene silencing in those cells [114]. Kooijmans et al. coated extracellular vesicles with nanobody-PEG-micelles, by which they improved poor cell specificity and increased circulation time *in vivo* [115].

Given the lack of pharmacological therapies for AKI, the kidney-targeted drug and siRNA delivery has drawn much attention in recent decades in order to improve the delivery efficacy in kidney. Although numerous carrier systems have been commonly used and shown promising effect in several diseases, only a few carrier systems like prodrugs and macromolecular carriers are the most frequently used strategies to achieve the goal of kidney-targeted drug delivery due to the size restriction of glomerulus filtration barrier. Drug targeting to the proximal tubule cells, which are the primary injured site in AKI could provide better treatment by means of enhancing the therapeutic efficacy of drugs and lowering unwanted side effects in other organs. In general, it only
allows carrier systems with a hydrodynamic diameter less than 10 nm to reach the site [9]. However, some studies also showed contradictory results that inorganic nanoparticles with the size ranging from 12-16 nm [22] and single-walled carbon nanotubes with average diameter less than 10 nm and average size 100 to 500 nm [28-31] can pass through the filtration threshold.

4.1 Polymeric Carrier Systems

Several studies have demonstrated that polymeric carriers had a potential to target kidney and might be good candidates for drug and siRNA delivery. The copolymers of N-vinylpyrrolidone with anionic maleic acid showed increased renal accumulation when compared with the unmodified polyvinylpyrrolidone (PVP) [116]. Another polymeric carrier that has widely been used for drug and siRNA targeting to kidney is low molecular weight chitosan (LMWC). 50% N-acetylated LMWC demonstrated the capability to specific target kidney [117, 118]. Afterwards, the LMWC-based carriers have been further explored and showed the potential for delivering drug and siRNA to kidney. Glucosamine, the fundamental unit of LMWC, showed enhanced protective effect against renal ischemia-reperfusion injury after conjugated to triptolide [119]. Chitosan/siRNA nanoparticles also demonstrated therapeutic effect against unilateral ureteral obstruction induced kidney injury [120].

4.1.1 Protein- and Peptide-based Carrier Systems

Low molecular weight proteins (LMWPs) and peptides were also extensively studied for renal delivery of therapeutic drugs. Lysozyme has a molecular weight of 14
kDa and can be freely filtered in the urine with a glomerular sieving coefficient of 0.8 [121], followed by the reabsorption by the proximal tubule cells. Therefore, lysozyme is one of the most popular protein carriers and has been conjugated to various drugs for specific renal delivery. For example, Prakash et al. has coupled Y27632 to lysozyme and they found that Y27632-lysozyme specifically accumulated in the kidney. The ischemia-reperfusion induced tubular damage was significantly inhibited after treatment of Y27632-lysozyme, which was indicated by decreased level of inflammation and fibrosis [122]. Although LMWPs showed promising ability as carrier systems for renal targeting, some defectiveness like cardiovascular toxicity and complexity in modification may hinder their applications.

Besides LMWPs, peptides are still widely studied in renal targeting drug delivery. Wischnjow et al. conjugated ciprofloxacin to the peptide-based drug carrier, (KKEEE)_3K, and successfully demonstrated the high selectivity to renal proximal tubule cells, indicating the excellent renal targeting potential of the peptide carrier [123]. Janzer and colleagues found that the targeting specificity of (KKEEE)_3K did not change after drug conjugation even with a multiple-drug loading of a model drug, α-lipoic acid. While other peptide molecules, such as (KKQQQ)_3K, showed a total loss of kidney specificity. This finding demonstrated (KKEEE)_3K as a promising carrier candidate for treatment of AKI [124].
4.1.2 Prodrugs

Prodrugs are compounds that can only be activated in the body after cleavage of chemical bonds [125-127]. Small drug molecules usually have poor chemical or metabolic stability, low water solubility and obvious toxicity in non-specific organs. Hence, development of prodrugs can improve these disadvantages and further give rise to patent line extension.

4.1.2.1 Folate Modified Prodrugs

Although the expression of folate receptors is well recognized in tumor cells [128, 129], the folate receptors are expressed in the kidneys as well. Folate can be filtered by glomerulus and reabsorbed by renal tubule cells as the form of 5-methylenetetrahydrofolate. Mathias and colleagues conjugated folic acid to diethylene triamine pentaacetic acid (DTPA) by an ethylenediamine spacer forming DTPA-folate conjugate [130]. They found the accumulation of the radiolabeled DTPA-folate in both kidneys was approximately 23% of the injected dose per gram tissue 4 h post intravenous injection. There was about 90% of DTPA-folate conjugates reduced in the kidneys after pretreatment of folic acid, which indicating that folate receptors in kidneys played an important role in the renal uptake of DTPA-folate conjugates. The other similar biodistribution study of $[^{99mTc}](CO)_{3}$-DTPA-folate demonstrated a higher renal accumulation which is about 50% of injected dose per gram tissue 4 h post intravenous injection [131]. However, the application of folate targeting in kidneys has been limited since folate receptors are not only expressed by kidneys [132].
4.1.2.2 Amino Acid Modified Prodrugs

Some endogenous enzymes have relatively high concentration in kidneys and hence their substrates, such as L-decarboxylation and γ-glutamyltranspeptidase, can be utilized to conjugate small drugs for targeting proximal tubule cells. Wilk and co-workers conjugate γ-glutamyl with dihydroxyphenylalanine to give γ-glutamyl-dopamine (GGDA) [133]. Its biodistribution study indicated that, compared with the administration of an equivalent dose of dopamine, an increased accumulation of dopamine in the kidney was observed following GGDA oral administration to mice. Even though dopamine has been proved to have no therapeutic effect on renal disease in clinic [134], this type of drug carrier can be conjugated with other drugs for renal targeting.

4.1.3 Nanoparticles

Besides above carrier systems, nanoparticles have a great potential as carriers for drug and siRNA delivery to kidney. Choi et al. demonstrated that nanoparticles with average hydrodynamic diameter of 75±25 nm have ability to target kidney mesangium by comparing renal distribution of polyethylene glycol (PEGylated) gold nanoparticles with different sizes [135]. Based on these findings, they first established the criteria of nanoparticles construction for targeting diseases associated with mesangium. Alidori and co-workers successfully delivered Trp53- and Mep1b-targeted siRNA to proximal tubule cells simultaneously using an ammonium-functionalized single-walled carbon nanotubes (fCNT) and mice lived longer with mitigated AKI [136]. In evaluation of pharmacokinetic profile of fCNT/siRNA nanoparticles in primates, it showed comparable to mice which would be a promising approach for clinical application. Another new finding broads the
effective treatment of AKI. Hu et al. designed dexamethasone-loaded E-selectin-targeting sialic acid-PEG-dexamethasone conjugate micelles and this nanoparticles showed enhanced kidney accumulation due to specific interaction between sialic acid and E-selectin, resulting in anti-inflammatory efficacy for acute kidney injury [137].

5. RNA Interference Therapy

RNA interference (RNAi) is a powerful gene silencing process which is guided by small RNAs including small interfering RNAs (siRNAs) and microRNAs (miRNAs) [138]. These small RNAs down regulate key gene expressions through sequence-specific degradation of mRNAs or inhibition of protein translation, by which the cell fate is determined [139-141]. In addition, the endogenous gene expression can also be suppressed by RNAi therapy [142, 143].

5.1 Small Interfering RNA

Small interfering RNAs (siRNA) consist of 20-25 base pairs and downregulate the expression of sequence-specific gene by post-transcriptional gene silencing pathway [144, 145]. Mechanistically, after entering cytoplasm siRNAs bind multiprotein RNA-inducing silencing complex (RISC) and then sequence-specifically target mRNA. The argonaute-2 which is also called slicer further triggers the cleavage of mRNA, leading to gene silencing. Unlike other gene technologies, siRNAs have several unique advantages, such as high degree of specificity to mRNAs, non-immunogenic property and high resistance to ribonucleases. With the development of technology for high-throughput gene expression and more understanding in several diseases, siRNAs have received increasing attention
and become one of the most promising therapeutic method for gene therapy [146, 147].

Soutschek and co-workers overcame the obstacle of in vivo gene silencing by intravenous administrating siRNAs which are chemically stabilized by modification of phosphorothioate backbone and 2’-O-methyl sugar on both sense and antisense strands [148]. They demonstrated decreased mRNA level of apolipoprotein B (apoB) in liver and jejunum, reduced plasma levels of apoB protein, as well as reduced total cholesterol. Moreover, they silenced human apoB in a transgenic mouse model, which indicates the promising potential for siRNAs in the treatment of human diseases.

5.2 Carrier Systems for siRNAs Delivery

The employment of naked siRNA is always associated with several inherent problems due to the poor cellular permeability, easy degradation by endogenous enzymes and off-target effects [149]. To further increase the delivery efficiency of siRNAs to the targeted tissues in vivo, a wide variety of carrier systems have been developed [150, 151]. Carrier systems can be divided into two categories: viral carriers and non-viral carriers. The viral carriers have some advantages including high transfection effect and long-term gene expression while disadvantages such as high toxicity, immunogenicity and economic issue hinder the application.

Non-viral carrier systems such as cationic lipids and cationic polymers have drawn much attention in improvement of gene therapies. These cationic delivery systems form polyelectrolyte complexes with the oppositely charged siRNA. In general, cationic lipids are made up with inner aqueous compartment which contains siRNAs and outer
phospholipid bilayer. Cationic polymers, like chitosan, polyamidoamine, polypeptides are chemically synthesized and form complexes with siRNAs. Reddy et al. encapsulated paclitaxel and siRNA targeting Bcl-2 into a nanocarrier system which consists of kojic acid backbone-based cationic amphiphile [152]. By doing so, they successfully inhibited cell proliferation and thus reduced tumor growth, demonstrating an efficient method for systemic delivery of siRNA. For another example, Dahlman et al. synthesized epoxide-modified lipid-polymer hybrids (7C1) for siRNA delivery to endothelium with high efficiency [153]. This formulation specifically reduced endothelial gene expression in lung to only 10% at a dose of 0.10 mg/kg and to 50% when the doses are as low as 0.02 mg/kg with no obvious gene expression decrease in other cells including luminary immune cells, hepatocytes and peritoneal immune cells. 7C1 nanoformulations should be a new candidate for siRNA delivery to endothelium in the future.

6. Chemokines and Chemokine Receptors

Chemokines, also known as chemotactic cytokines, are small heparin-binding signaling proteins secreted by various stromal and epithelial cells and able to direct the movement of circulating leukocytes to inflammatory or injured sites via interacting with their respective chemokine receptors [154]. Chemokines and their receptors are well recognized as contributors for the pathogenesis of several diseases. And blockade of chemokine receptors has been used to regulate inflammation in many diseases. There are approximately 50 human chemokines which can be divided into four groups based on the number and spacing of N-terminal cysteines (Table 2) [155-157]. Among chemokines, CC chemokines are the largest family and have four cysteine residues with first two adjacent
to each other. CXC chemokines are the chemokines that have a single amino acid residue within the first two canonical cysteines. Fractalkine [158, 159] and lymphotactin [160] is the only member of the other two families, CX3C and XC respectively. There are 19 different chemokine receptors that all belong to the seven-transmembrane G-protein-coupled receptor family. Chemokines bind to their correspondent chemokine receptors on cell surface, by which signaling cascades are activated leading to cell rearrangement, change of shape or cell movement of action.
<table>
<thead>
<tr>
<th>Chemokine Family</th>
<th>Receptor</th>
<th>Chemokine Ligands</th>
<th>Cell Types</th>
<th>Disease Connection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>CCL3 (MIP-1α), CCL5 (RANTES), CCL7 (MCP-3), CCL14 (HCC1)</td>
<td>T cells, monocytes, eosinophils, basophils</td>
<td>Rheumatoid arthritis, multiple sclerosis</td>
<td></td>
</tr>
<tr>
<td>CCR2</td>
<td>CCL2 (MCP-1), CCL8 (MCP-2), CCL7 (MCP-3), CCL13 (MCP-4), CCL16 (HCC4)</td>
<td>Monocytes, dendritic cells (immature), memory T cells</td>
<td>Atherosclerosis, rheumatoid arthritis, multiple sclerosis, resistance to intracellular pathogens, type 2 diabetes mellitus</td>
<td></td>
</tr>
<tr>
<td>CCR3</td>
<td>CCL11 (eotaxin), CCL13 (eotaxin-2), CCL7 (MCP-3), CCL5 (RANTES), CCL8 (MCP-2), CCL13 (MCP-4)</td>
<td>Eosinophils, basophils, mast cells, Th2, platelets</td>
<td>Allergic asthma and rhinitis</td>
<td></td>
</tr>
<tr>
<td>CCR4</td>
<td>CCL17 (TARC), CCL22 (MDC)</td>
<td>T cells (Th2), dendritic cells (mature), basophils, macrophages, platelets</td>
<td>Parasitic infection, graft rejection, T-cell homing to skin</td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>CCL2 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES), CCL11 (eotaxin), CCL14 (HCC1), CCL16 (HCC4)</td>
<td>T cells, monocytes</td>
<td>HIV-1 coreceptor (T-tropic strains), transplant rejection</td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>CCL20 (MIP-3γ), LARC</td>
<td>T cells (T regulatory and memory), B cells, dendritic cells</td>
<td>Mucosal humoral immunity, allergic asthma, intestinal T-cell homing</td>
<td></td>
</tr>
<tr>
<td>CCR7</td>
<td>CCL19 (ELC), CCL21 (SLC)</td>
<td>T cells, dendritic cells (mature)</td>
<td>Transport of T cells and dendritic cells to lymph node, antigen presentation, and cellular immunity</td>
<td></td>
</tr>
<tr>
<td>CCR8</td>
<td>CCL1 (I309)</td>
<td>T cells (Th2), monocytes, dendritic cells</td>
<td>Dendritic-cell migration to lymph node, type 2 cellular immunity, granuloma formation</td>
<td></td>
</tr>
<tr>
<td>CCR9</td>
<td>CCL25 (TECK)</td>
<td>T cells, IgA+ plasma cells</td>
<td>Homing of T cells and IgA+ plasma cells to the intestine, inflammatory bowel disease</td>
<td></td>
</tr>
<tr>
<td>CCR10</td>
<td>CCL27 (CTACK), CCL28 (MEC)</td>
<td>T cells</td>
<td>T-cell homing to intestine and skin</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. CC, CXC, CX3C and XC Families of Chemokines and Chemokine Receptors

(Adapted from [161]. Reproduced with permission from scientific reference citation, Copyright Massachusetts Medical Society).
Continued Table 2. CC, CXC, CX3C and XC Families of Chemokines and Chemokine Receptors (Adapted from [161]. Reproduced with permission from scientific reference citation, Copyright Massachusetts Medical Society).

<table>
<thead>
<tr>
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<th>Chemokine Ligands</th>
<th>Cell Types</th>
<th>Disease Connection</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXC Family</td>
<td>CXCR1</td>
<td>CXCL8 (interleukin-8), CXCL6 (GCP2)</td>
<td>Neutrophils, monocytes</td>
<td>Inflammatory lung disease, COPD</td>
</tr>
<tr>
<td></td>
<td>CXCR2</td>
<td>CXCL8, CXCL1 (GROα), CXCL2 (GROβ), CXCL3 (GROγ), CXCL5 (ENA-78), CXCL6</td>
<td>Neutrophils, monocytes, micro-vascular endothelial cells</td>
<td>Inflammatory lung disease, COPD, angiogenic for tumor growth</td>
</tr>
<tr>
<td></td>
<td>CXCR3-A</td>
<td>CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)</td>
<td>Type 1 helper cells, mast cells, mesangial cells</td>
<td>Inflammatory skin disease, multiple sclerosis, transplant rejection</td>
</tr>
<tr>
<td></td>
<td>CXCR3-B</td>
<td>CXCL4 (PF4), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC)</td>
<td>Microvascular endothelial cells, neoplastic cells</td>
<td>Angiostatic for tumor growth</td>
</tr>
<tr>
<td></td>
<td>CXCR4</td>
<td>CXCL12 (SDF-1)</td>
<td>Widely expressed</td>
<td>HIV-1 coreceptor (T-cell-tropic), tumor metastases, hematopoiesis</td>
</tr>
<tr>
<td></td>
<td>CXCR5</td>
<td>CXCL13 (BCA-1)</td>
<td>B cells, follicular helper T cells</td>
<td>Formation of B-cell follicles</td>
</tr>
<tr>
<td></td>
<td>CXCR6</td>
<td>CXCL16 (SR-PSOX)</td>
<td>CD8+ T cells, natural killer cells, and memory CD4+ T cells</td>
<td>Inflammatory liver disease, atherosclerosis (CXCL16)</td>
</tr>
<tr>
<td>CX3C Family</td>
<td>CX3CR1</td>
<td>CX3CL1 (fractalkine)</td>
<td>Macrophages, endothelial cells, smooth-muscle cells</td>
<td>Atherosclerosis</td>
</tr>
<tr>
<td>CX Family</td>
<td>XCR1</td>
<td>XCL1 (lymphotactin), XCL2</td>
<td>T cells, natural killer cells</td>
<td>Rheumatoid arthritis, IgA nephropathy, tumor response</td>
</tr>
</tbody>
</table>
6.1 CXCR4 and its Ligand SDF-1

C-X-C chemokine receptor 4 (CXCR4) is one of the most commonly expressed chemokine receptors in many tissues, which plays a vital role in the process of metastasis in multiple types of cancers and inflammatory diseases [162-165]. Binding with its ligand, stromal cell-derived factor-1 (SDF-1), divergent intracellular signaling transduction pathways are initiated, which can result in a variety of responses such as chemotaxis, cancer cell proliferation, recirculation and hematopoiesis of leukocytes [166, 167]. Driven by CXCR4 expressed in the primary tumor cells and leukocytes, the cells metastasize to secondary sites where SDF-1 is highly expressed, such as lung, liver, bone marrow, and brain [168]. For example, phosphatidylinositol-3-kinase (PI3K) pathway is activated by CXCR4/SDF-1 axis, leading to activation of protein kinase AKT, which is the key mediator in cancer cell migration and survival [169]. Moreover, the secretion of matrix metalloproteinases (MMPs) is increased and then extracellular matrix further degrades after CXCR4 activation, which facilitates the invasion process. Mitogen-activated protein kinase (MAPK) pathways is stimulated by CXCR4/SDF-1 axis as well, resulting in increase in cancer cell proliferation and survival [170]. Blocking the CXCR4/SDF-1 interaction by antagonizing CXCR4 can inhibit macrophage infiltration, induce tumor growth arrest and prevent metastatic spread [171, 172].

6.2 The Role of CXCR4 in AKI

Inflammation is a significant pathophysiological component of AKI [164, 173]. Cytokines and chemokines are involved in response to nephrotoxicity or reperfusion injury, leading to a proinflammatory microenvironment. CXCR4 and its ligand SDF-1 are
believed to be involved in recirculation and hematopoiesis of normal leukocytes, including neutrophils, monocytes and lymphocytes [167, 172, 174], which are key mediators of renal injury. Sustained high CXCR4 expression (on tubules or immune cells, such as macrophages), in turn, exacerbates renal injury and promotes necrosis. Inhibiting CXCR4 has been demonstrated to modulate immune system, leading to improved therapeutic activity of AKI [173, 175]. Zuk and co-workers showed renoprotective role of plerixafor (AMD3100) which is a small-molecule CXCR4 antagonist in renal ischemia-reperfusion animal model [173]. After antagonizing CXCR4, plerixafor ameliorates AKI by modulating leukocyte infiltration and reducing expression of proinflammatory chemokines and cytokines.
Chapter 2. Determinants of synthetic polymeric carrier systems for renal-targeting in acute kidney injury

1. Introductions

Drug targeting to the proximal tubule cells, which are the primary injured site in AKI could provide better treatment by means of enhancing the therapeutic efficacy of drugs and lowering unwanted side effects in other organs. Synthetic polymers are often used as carriers to target proximal tubular cells by means of either physically encapsulate or covalently conjugate drugs [176-178]. In recent years, several studies have been demonstrated that polymers have potential to target proximal tubular cells and might be good candidate for drug carriers. Kamada et al. demonstrated that polyvinylpyrrolidone-co-dimethyl maleic anhydride [poly(VP-co-DMMAn)] had much higher accumulation and longer retention time in kidneys than native polyvinylpyrrolidone (PVP). The poly(VP-co-DMMAn)-modified superoxide dismutase also accelerated recovery from mercuric chloride-induced acute renal failure [179]. Mitra et al studied the biodistribution of N-(2-Hydroxypropyl) Methacrylamide (HPMA) copolymers with different molecular weight and charge [180]. They synthesized neutral and negatively charged HPMA copolymers with average molecular weights of 7 kDa, 21 kDa and 70 kDa. The negatively charged 21 kDa HPMA copolymers showed highest renal accumulation 24 h after intravenous injection in mice. These findings proved that the type and content of anionic groups introduced to the polymers, as well as polymer molecular weight play a vital role for renal cells targeting. However, the reported studies were performed in healthy
kidneys and very little is currently known about how physicochemical properties of polymers affect renal deposition in AKI.

Therefore, we synthesized and evaluated a library of polymers with a range of molecular weights and different net charges. We compared the cellular uptake and intracellular trafficking of polymers in mouse proximal tubular epithelial (MCT) cells at both normoxia and hypoxia conditions. Additionally, by testing biodistributions of each polymers in vivo, we explored and identified preferred polymer characteristics that preferentially accumulate in proximal tubular cells in AKI animal model.

2. Materials and Methods

2.1 Materials

N-(3-Aminopropyl) methacrylamide hydrochloride (APMA) and N-(2-hydroxypropyl) methacrylamide (HPMA) were purchased from Polysciences, Inc. (Warrington, PA). Methacrylic acid (MAA) was obtained from Acros Organics (Belgium, NJ). 4, 4′-Azobis(4-cyanovaleric acid) (ACVA), 4-cyano-4-(phenyl carbonothioylthio) pentanoic acid (CPAD) which was as a typical CTA and fluorescein O-methacrylate (FMA) were from Sigma-Aldrich (St. Louis, MO). Cyclam (1,4,8,11-tetraazacyclotetradecane) was purchased from Alfa Aesar (Ward Hill, MA). N,N′-hexamethylenebisacrylamide (HMBA) was from Polysciences, Inc. (Warrington, PA). AMD3100 (base form) was from Biochempartner (Shanghai, China). RPMI medium 1640, Dulbecco’s phosphate buffered saline (PBS), and fetal bovine serum (FBS) were from Thermo Scientific (Waltham, MA). Mouse renal tubular epithelial cells (MCT) was a kind gift from Dr. Padanilam (University
of Nebraska Medical Center) and cultured in RPMI supplemented with 10% FBS. All other reagents were from Fisher Scientific and used as received unless otherwise noted.

2.2 Methods

2.2.1 Synthesis of Polymers

The polymerization was achieved at 70 °C, employing ACVA as the initiator and CPAD as the chain transfer agent. A typical protocol is as follows: APMA (178 mg, 1 mmol) was dissolved in doubly distilled water followed by the addition of CPAD (6.24 mg, 0.02 mmol, target DP \( n = 45 \)), ACVA (2 mg, 0.007 mmol) and FMA (7.15 mg, 0.018 mmol) in 1,4-dioxane stock solution. The solution was added into a small glass vial and purged with nitrogen for at least 30 minutes. The vial was placed in an oil bath for polymerization at 70 °C for 30 min, 3 h, and 4.5 h. After the polymerization, the solvent was dialyzed against water for 3 days before final freeze-drying.

The polymerization of pMAA and pHpMA was conducted employing similar procedure described above. The feed ratio, reagent and reaction time was according to Table 2.

2.2.2 Polymer Characterizations

The molecular weights of pAPMA were analyzed by gel permeation chromatography (GPC) operated in 0.1 M sodium acetate buffer (pH 5.0) using Agilent 1260 Infinity LC system equipped with a miniDAWN TREOS multi-angle light scattering (MALS) detector and a Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The column used was TSKgel G5000PWXL-CP (Tosoh Bioscience LLC, King of Prussia, PA) at a flow rate of 0.5 mL/min. Results were analyzed using Astra 6.1
software from Wyatt Technology. The degree of polymerization was calculated from
the GPC.

The characterization of pMAA was conducted employing the same method but
using AquaGel PAA-202 (London, ON, Canada) column. The characterization of pHHPMA
was conducted employing the same method but using 0.3 M sodium acetate buffer (pH
5.0).

The amount of conjugated FMA was determined on a SpectraMax iD3 Multi-Mode
Microplate Reader (Molecular Devices, CA). An amount of 1 mg of each dried polymers
was dissolved in 1 ml PBS and the fluorescence intensity of the solution was measured at
an excitation wavelength of 490 nm and an emission wavelength of 525 nm, which
corresponds to a peak maximum for FMA. FMA content was determined by comparison
to a FMA standard curve.

2.2.3 Cellular Uptake and Intracellular Trafficking of Polymers

Flow cytometry analysis was conducted to study the cellular uptake of polymers.
MCT cells (3 × 10^4) were seeded in 12-well plates and cultured to approximately 50%
confluence. The cells were incubated in 37 °C at a virtual pAPMA concentration of 2
µg/mL, pMAA concentration of 300 µg/mL or pHHPMA concentration of 1 mg/mL for 24 h
in either normoxic or hypoxic (2% O2) incubator. The cells were then washed with PBS
twice, trypsinized and subjected to analysis using a BD FACS Calibur flow cytometer (BD
Bioscience, Bedford, MA). The results were processed using FlowJo software.

Intracellular trafficking was observed by LSM 800 Laser Scanning Microscope (Zeiss,
Jena, Germany). MCT cells were cultured on 24-well plates with round coverslip glass at
5 × 10^4 cells/well. After 24 h, the medium was exchanged with fresh medium and a solution of the studied polymers was added (same concentrations as cellular uptake). After incubation for another 24 h in either normoxia or hypoxia incubator, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min and nuclei were stained with Hoechst 33258 for another 10 min.

2.2.4 In vitro Cytotoxicity of the Polymers

Toxicity of the polymers was evaluated by Cell Titer Blue assay in MCT cells. The cells (5 X 10^3 cells/well) were plated in 96-well microplates. After 24 h, the cultured cells were treated with fresh medium and different polymers respectively. After further 24 h of incubation at either normoxic or hypoxic condition, the medium was removed and replaced with a mixture of 100 µL of serum-free media and 20 µL of CellTiter-Blue reagent (CellTiter-Blue Cell Viability Assay, Promega). After 2 h of incubation, the fluorescence (560/590 nm) was measured on a SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Devices, CA). The relative cell viability (%) was calculated as 

\[
\frac{\text{[fluorescence] sample}}{\text{[fluorescence] untreated}} \times 100.
\]

2.2.5 Induction of Ischemia-reperfusion Kidney Injury

To induce AKI, we used the animal model of unilateral or bilateral renal ischemia-reperfusion (I/R) injury. All animal experiments followed a protocol approved by the UNMC Institutional Animal Care and Use Committee. C57BL/6J male mice (8-10 week old) were purchased from Charles River. All animals were given free access to food and water. The non-fluorescent food was given at least 1 week before surgery. The mice were anesthetized by intraperitoneal administration of ketamine (200 mg/kg) and xylazine (16
mg/kg). For unilateral I/R injury, following left dorsal flank incision, renal pedicles were bluntly dissected and a microvascular clamp (Roboz Surgical Instrument, Gaithersburg, MD) was placed on the left renal pedicle for 30 min to induce unilateral I/R injury. During the procedure, mice were kept well hydrated with warm saline and on 37 °C heating pad. After 30 min of occlusion, the clamps were removed and kidney reperfusion was verified visually. Then wound clips were used to close skin. Sham-operated right side underwent the same surgical procedure, except for the occlusion of the renal arteries. The mice were monitored until they woke up. Similar procedure was conducted to induce bilateral I/R injury by placing microvascular clamp on both renal pedicle for 30 min. Sham-operated control animals also underwent the same surgical procedure, except for the occlusion of the renal arteries.

2.2.6 In vivo Biodistribution of Polymers

Biodistribution of the polymers in I/R mice was analyzed by ex vivo fluorescence imaging. Each different polymers were administered via tail vein injection 24 h post-surgery. At 4 h and 24 h after administration, mice were killed and major organs were isolated and imaged using Xenogen IVIS 200. Emission wavelength of 540 nm and excitation wavelength of 500 was used to image organs. The fluorescence intensities from liver and kidney was quantified using Living Image® 4.5 software. The radiant efficiency of the kidney and liver was measured as (photons/sec/cm²/sr)/(µW/cm²). Background fluorescence was subtracted prior to analysis. To study intracellular localization of different polymers in kidney, bisected kidneys were embedded in OCT compound and cut into 10 µm frozen sections. The frozen sections were either stained with DAPI, or
stained with haematoxylin and eosin (H&E). Polymer localizations were visualized by LSM 800 Laser Scanning Confocal Microscope (Zeiss, Jena, Germany). Histopathological change was confirmed by H&E sections under EVOS microscope.

2.2.7 Tissue Homogenization

To quantify the amounts of polymers in I/R kidneys and sham-operated kidneys, kidneys dissected from C57BL/6J mice bodies were finely minced with scissors and placed in a homogenizer vessel. 750 µL RIPA buffer and Halt™ protease & phosphatase inhibitor cocktail were added and tissues were subsequently homogenized. Homogenized samples were centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was used to quantify polymer amount in kidneys by standard addition method using a fluorescence Synergy 2 Microplate Reader (BioTek, VT).

2.2.8 Statistical Analysis

Data are presented as the means ± SD. The statistical significance of the mean values of all groups was determined by using one-way ANOVA. An unpaired t test was used to compare the means of two different groups. A P value < 0.05 was considered statistically significant.

3. Results and Discussions

3.1 Polymer Synthesis and Characterization

Polymers such as HPMA, APMA or MAA have been used as carriers of drugs and siRNA to enhance their stability in vivo and as targeted delivery systems. Lots of low-molecular weight therapeutic agents show improved activity when conjugated to or encapsulated into carrier systems containing such polymers [181-183]. Because the
biodistribution of such therapeutic agents is influenced by the properties of the carrier system, we developed a library of 9 fluorescently labeled polymers, including neutral pHMPA, negatively charged pMAA, and positively charged pAPMA, with reversible addition-fragmentation chain-transfer (RAFT) polymerization method to evaluate properties both *in vitro* and *in vivo*. Table 3 shows the RAFT polymerization condition of pAPMA, pMAA and pHMPA, in which polymers are prepared by copolymerization of the corresponding monomers with a fluorescein-containing co-monomer using CTA transfer agent and ACVA initiator at 70°C. The feed ratio of monomer/CTA/ACVA/FMA is variable according to different monomers. All polymers are characterized by GPC. The molecular weight range is from 5 kDa to 36 kDa with a low Mw/Mn value (Table 3). The fluorescence of the synthesized polymers is used to track the subcellular fate and distribution of the polymers both *in vitro* and *in vivo*. The content of FMA is quantified for each polymers (Table 3) in order to enable the comparison of fluorescence data obtained from the different polymers. The GPC curves for all polymers are shown in Figure 1.
Table 3. Polymer Characterizations

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Feed ratio</th>
<th>Reagent</th>
<th>Time (h)</th>
<th>Mn</th>
<th>Mw/Mn</th>
<th>mol% FMA/polymer in feed</th>
<th>mg% FMA/polymer in polymer</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAPMA-6</td>
<td>150:3:1 1: 2.7</td>
<td>1,4-dioxane</td>
<td>0.5</td>
<td>6,110</td>
<td>1.17</td>
<td>1.8</td>
<td>8.35</td>
</tr>
<tr>
<td>pAPMA-16</td>
<td>150:3:1 1: 2.7</td>
<td>1,4-dioxane</td>
<td>3</td>
<td>16,450</td>
<td>1.12</td>
<td>1.8</td>
<td>4.02</td>
</tr>
<tr>
<td>pAPMA-30</td>
<td>150:3:1 1: 2.7</td>
<td>1,4-dioxane</td>
<td>4.5</td>
<td>30,490</td>
<td>1.13</td>
<td>1.8</td>
<td>6.03</td>
</tr>
<tr>
<td>pMMAA-5</td>
<td>175:3:1 1:1.7</td>
<td>Methanol</td>
<td>24</td>
<td>5,170</td>
<td>1.09</td>
<td>0.86</td>
<td>2.08</td>
</tr>
<tr>
<td>pMMAA-16</td>
<td>352:3:1 1:3.4</td>
<td>Methanol</td>
<td>24</td>
<td>16,340</td>
<td>1.05</td>
<td>0.86</td>
<td>0.60</td>
</tr>
<tr>
<td>pMMAA-31</td>
<td>704:3:1 1:5.8</td>
<td>Methanol</td>
<td>24</td>
<td>31,390</td>
<td>1.02</td>
<td>0.86</td>
<td>0.54</td>
</tr>
<tr>
<td>pHMPA-5</td>
<td>103:3:1 1:1.4</td>
<td>Methanol</td>
<td>24</td>
<td>5,510</td>
<td>1.19</td>
<td>1.4</td>
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<tr>
<td>pHMPA-16</td>
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<td>Methanol</td>
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<td>15,790</td>
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<tr>
<td>pHMPA-36</td>
<td>400:3:1 1:5.6</td>
<td>Methanol</td>
<td>24</td>
<td>36,820</td>
<td>1.05</td>
<td>1.4</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Table 3. Polymer Characterizations

a. Initial molar ratio of monomer/CTA/ACVA/FMA
Figure 1. GPC curves of all polymers.
3.2 Cellular Uptake and Intracellular Trafficking of Polymers

For many applications of RAFT generated polymers in biopharmaceutical use, potential for toxicity is a concern. Therefore, it is important to understand toxicity of polymers in MCT cells, mouse renal proximal tubule cells, before conducting cellular uptake. To evaluate cytotoxicity of polymers, CellTiter Blue assay was conducted in MCT cells (Table 4). Because we used unilateral renal ischemia-reperfusion injury model (explained below) to study biodistribution of polymers in vivo, we evaluated cytotoxicity at both normoxia and hypoxia conditions. We found there was no obvious toxicity after we incubated MCT cells with pHMA at 1 mg/mL at both normoxia and hypoxia regardless of size. The HPMA based copolymers have been extensively examined as biocompatible, non-immunogenic and non-toxic drug carriers [184, 185]. For pMAA and pAPMA, generally the smaller was the size, the less toxic was the polymers. And IC₅₀ at hypoxia was generally lower than normoxia, which may result from more cellular uptake of polymers at hypoxia. pAPMA showed much higher toxicity to MCT cells due to highly positive charges.

In order to compare uptake of polymers in vitro at normoxia and hypoxia, we investigated the cellular uptake of different polymers. MCT cells were selected and incubated with the polymers for 24 h and cellular uptake was determined by flow cytometry. The mean fluorescence intensity (MFI) ratio between normoxia and hypoxia was showed in Figure 2. For pAPMA, cells had similar MFI after incubated at either normoxia or hypoxia. Among pMAA, the fold of MFI for pMAA-5 was ~ 2.8, while pMAA-16 and pMAA-31 didn’t show much diverse cell uptake at normoxia and hypoxia.
For pHpMA with all three sizes, more polymers were taken up by MCT cells at hypoxia and the highest ratio was observed with pHpMA-36, which was about 4 folds.

Flow cytometry yields semi-quantitative results for fluorescence taken up by cells or attached to their outer membrane, therefore we used confocal laser scanning microscopy to verify intracellular trafficking of each polymers. Similarly, the cells were incubated with different polymers for 24 h and then imaged by confocal microscopy (Figure 3). All polymers were clearly observed inside the cells. They were internalized into the cells and mainly localized in the cytoplasm. Compared with normoxia, there was no difference observed for cellular trafficking of pAPMA at hypoxia. For pMAA, a slightly higher intensity was only observed for pMAA-5 after hypoxic incubation. However, the fluorescence intensities in cytoplasm of pHpMA with all sizes were significantly increased after cells were incubated at hypoxia. And the most significant difference was also observed with pHpMA-36. These results of cellular uptake and intracellular trafficking indicated that MCT cells had increased cell uptake at hypoxia for certain polymers, such as smallest sized pMAA (pMAA-5) and those three pHpMA polymers at hypoxia, which prompted us to further investigate the distribution in vivo.
<table>
<thead>
<tr>
<th>Polymers</th>
<th>IC50 [µg/ml]</th>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAPMA-6</td>
<td>10.6</td>
<td>4.7</td>
<td></td>
</tr>
<tr>
<td>pAPMA-16</td>
<td>7.9</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>pAPMA-30</td>
<td>8.5</td>
<td>7.9</td>
<td></td>
</tr>
<tr>
<td>pMAA-5</td>
<td>1000.2</td>
<td>890.4</td>
<td></td>
</tr>
<tr>
<td>pMAA-16</td>
<td>859.5</td>
<td>657.0</td>
<td></td>
</tr>
<tr>
<td>pMAA-31</td>
<td>613.8</td>
<td>496.1</td>
<td></td>
</tr>
<tr>
<td>pHMA-5</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>pHMA-16</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>pHMA-36</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.** IC50 of different polymers in MCT cells at normoxia or hypoxia condition. Cell viability was measured by CellTiter blue after 24 h incubation at 37 °C.
**Figure 2.** Cellular uptake of different polymers in MCT cells. Quantification of cellular uptake is shown by mean fluorescence intensity (MFI) ratio between cells incubated at hypoxia and at normoxia after 24 h. Data were shown as the mean ± SD (n = 3).
**Figure 3.** Intracellular trafficking of different polymers in MCT cells by confocal microscopy after 24 h incubation (63X).
3.3 Renal accumulation of polymers in unilateral I/R mice

Polymer size and charge play an important role in kidney deposition because glomerulus basement membrane filters small molecules by size and charge. Generally, in healthy state, molecules with hydrodynamic diameter less than 5-7 nm or molecular weight less than 68 kDa can pass this barrier. However, the barrier can be broken by podocyte effacement in disease and large or charged molecules could accumulate in the Bowman space due to the leaky and abnormal fenestrae after renal ischemia [186-188]. Therefore, we hypothesized that renal targeting of polymers can be impacted by AKI and this impact can be associated with size or charge of polymers.

To understand how AKI affects the biodistribution, especially for renal accumulation, of polymers with different sizes and charges, we established a well-known kidney injury model, unilateral ischemia-reperfusion (I/R) model, where left renal artery was clamped and renal injury was induced in the left kidney while the right kidney was also exposed and remained intact. Mice were sacrificed at two designated time points, 4 h or 24 h post intravenously administration and major organs were isolated for ex vivo fluorescence imaging (Figure 4A). Generally, we didn’t find any less polymers entering into I/R kidneys due to reduced blood perfusion as compared to sham-operated kidneys at these two time points. In contrast, we found polymers with certain size and charge showed relative more accumulation in I/R kidneys at 24 h. Among negatively charged pMAA and neutral pHMA, pMAA-5 and pHMA-36 showed most distinct accumulation in I/R and sham-operated kidneys at 24 h. Region of interest (ROI) ratio between I/R and sham-operated kidneys of these two polymers at 24 h was ~ 4, and the
renal accumulation was similar at 4 h (Figure 4B). The higher the molecular weight of pMAA (pMAA-16 and pMAA-31) or the lower the molecular weight of pHMA (pHPMA-5 and pHPMA-16), the less the fluorescence difference between I/R and sham-operated kidneys at 24 h. Sluggish and occasionally retrograde blood flow after I/R injury and delayed clearance of macromolecules from the injured kidneys may lead to the delayed and increased accumulation at 24 h [189, 190]. Pathological change of kidneys after I/R injury, e.g. endothelial dysfunction, can also contribute to change in interaction between macromolecules and kidneys at cellular level [189, 191]. These polymers did not appear to be taken up to a significant extent by any organ other than kidneys, especially for the two major organs of reticuloendothelial system, liver and spleen (Figure 4A & 6C). In contrast, for positively charged polymers pAPMA, we didn’t observe any obvious difference between I/R and sham-operated kidneys, except for high liver accumulation of pAPMA-16 at 4 h and pAPMA-30 at 24 h. Positively charged macromolecules are usually susceptible to rapid renal elimination and high non-specific uptake in liver [23, 24]. These results indicated that AKI has size-, and charge-dependent effect on renal uptake of synthetic polymers. Its potential to take more pMAA-5 and pHMA-36 may provide us a new angle of view to design polymeric carrier systems for renal targeting in AKI.

To quantify the amount of polymers in I/R and sham-operated kidneys and make sure that the fluorescence observed from IVIS was from fluorescent labeled polymers, instead of the auto-fluorescence of the tissues, plate-reader was used to detect fluorescence in the tissue homogenates. Considering the possible auto-fluorescence from these tissues, we analyzed these data using standard addition (Figure 5). On the basis of
homogenized tissues, it is evident that the ratio of each polymers amount between I/R and sham-operated kidneys was consistent with the ROI ratio from IVIS results shown above. The highest ratio of polymers between I/R and sham-operated kidneys was observed in pMAA-5 and pHMA-36, which accorded with previous IVIS results.

To further understand this selective accumulation of polymers with different sizes and charges in I/R kidney, we conducted intracellular localization and histological studies on the both I/R and sham-operated kidney with frozen sections and H&E staining, respectively (Figure 6). In sham-operated kidneys, even though kidneys had been exposed to polymers, kidney tissues remained normal. For I/R kidneys, extensive tubular dilatation and cast formation were found in cortex. According to confocal pictures, we found in I/R and sham-operated kidneys all the 9 polymers passed glomerular filtration barrier into Bowman space and finally can be taken up by proximal tubule cells in some extent. The molecular weights of these polymers are less than 68 kDa which is a threshold for macromolecules to cross this barrier. Even though basement membrane has negative charge, it does not impact on the filtration of these polymers in either I/R or sham-operated kidneys. When getting into Bowman space, majority of positively charged pAPMA may be susceptible to rapid renal clearance and be quickly eliminated from kidney, while the excretion of negatively charged pMAA and neutral pHMA from cortex to renal pelvis might be limited in I/R kidneys [189, 192] and such polymers can be selectively reabsorbed and sustained by renal tubule cells which are damaged during AKI.
**Figure 4.** Biodistribution of pAPMA, pMAA and pHMA in mice with unilateral I/R injury at the indicated time points. (A) Ex vivo imaging of dissected organs at 4 or 24 h after administration, which indicating lung, heart, liver, spleen, left kidney and right kidney (from left to right and top to bottom). Region-of-interest (ROI) ratios of left to right kidney (B), or ROI ratio of left kidney to liver (C) at indicated time points were quantified. Data were shown as the mean ± SD (n = 5). ***, p < 0.01.
Figure 5. Quantification of different polymers in left and right kidneys. Kidneys were homogenized and the fluorescence of each sample was measured by standard addition method. A-C) pAPMA-6, pAPMA-16 and pAPMA-30; D-F) pMAA-5, pMAA-16 and pMAA-31; G-I) pHPMA-5, pHPMA-16 and pHPMA-36. Data are shown as mean ± SD (n = 5).
Figure 6. Confocal microscopy images (20X) and H&E staining (20X) of kidney sections. Kidneys were extracted from mice with unilateral I/R injury receiving pAPMA-6, pAPMA-16 or pAPMA-30.
Continued Figure 6. Confocal microscopy images (20X) and H&E staining (20X) of kidney sections. Kidneys were extracted from mice with unilateral I/R injury receiving pMAA-5, pMAA-16 or pMAA-31.
Continued Figure 6. Confocal microscopy images (20X) and H&E staining (20X) of kidney sections. Kidneys were extracted from mice with unilateral I/R injury receiving pHMA-5, pHMA-16 or pHMA-36.
3.4 Validation of Renal Accumulation in Bilateral I/R Kidney Injury Model

Based on previous results, we had two concerns. First, are these results reproducible? Second, is unilateral I/R injury model comparable to bilateral renal I/R injury model in this study? Bilateral renal I/R injury model is most frequently used in experimental study because it is a clinically relevant model to study the AKI. However unilateral renal I/R injury animal model provides us lots of benefits in renal accumulation study. For example, it can avoid individual difference and reduce the risk of mortality due to the functional redundancy [193]. To verify that our results are also applicable to bilateral I/R animal model as well as repeat the results, we selected two polymers, pAPMA-30 and pHHPMA-16. We selected pAPMA-30 because we didn’t find any effect of AKI on its accumulation in kidneys. On the contrary, pHHPMA-16 in I/R kidneys was ~3.5 times as much as it was in sham-operated kidneys (Figure 4B & 5H). Considering the relatively large standard deviation in pHHPMA-36 (Figure 4B), we did not select it although the variation of fluorescence intensity between I/R and sham-operated kidneys was slightly bigger. To evaluate renal accumulation of pAPMA-30 and pHHPMA-16 in bilateral I/R animal model, similar procedure was performed. As demonstrated in Figure 7B, a strong fluorescent signal was observed in I/R kidneys after administration of pHHPMA-16 at 4 h and it was sustained at 24 h. Similar to the result from unilateral I/R model, pAPMA-30 showed no preference to accumulate in the I/R kidneys at both 4 h and 24 h (Figure 7A). In addition, compared with sham-operated kidneys, quantified fluorescence intensity of I/R kidneys increased to ~2.1 folds and ~2.5 folds for pHHPMA-16 at 4 h and 24 h, respectively, on the basis with ROI ratio. Alteration in renal hemodynamics for unilateral and bilateral renal
I/R injury model might contribute to the differences in renal accumulation of pHMA-16 at 4 h. But parallel results were seen in these two animal models that polymers potentially accumulate in I/R kidneys in unilateral renal I/R model also tend to accumulate in I/R kidneys in bilateral renal I/R model, and vice versa. Hence, this study confirmed our previous results and indicated that unilateral I/R animal model can be a viable model for us to investigate renal accumulation of polymers in AKI.
**Figure 7.** Biodistribution of pAPMA-30 and pHMA-16. I/R mice or sham-operated mice were given one injection of pAPMA-30 or pHMA-16 at 24 h post-surgery. Ex vivo imaging of pAPMA-30 (A) and pHMA-16 (B) were taken at 4 h or 24 h after administration. Organs were lung, heart, liver, spleen, left kidney and right kidney (from left to right and top to bottom). (C) Quantified ratio of ROI between kidneys from I/R mice and sham-operated mice. Data are shown as mean ± SD (n = 3).
4. Conclusion

In summary, unilateral renal I/R injury model allowed us to obtain the understanding that AKI alters renal accumulation of polymers with various sizes and charges. Our data suggested that polymers that showed the most enhanced ability to accumulate in I/R kidneys were negatively charged pMAA-5 and neutral pHPMA-36. These new findings provide not only advance our fundamental understandings of transport of polymeric drug delivery systems in normal and injured kidneys, but also initial insights on the polymeric drug delivery systems to AKI.

1. Introduction

Acute kidney injury (AKI) is characterized by a sudden loss of renal function with high morbidity and mortality [42, 194, 195]. AKI affects over 13 million patients worldwide every year and remains a global public health concern [35]. It is a difficult-to-prevent medical problem which involving complexed biological process, such as vasoconstriction, leukostasis, immune activation and apoptosis [56, 196, 197]. AKI may finally lead to chronic kidney disease and ultimately kidney failure [40], in which case the patient would require expensive renal dialysis or transplantation [198].

As opposed to treating the damage per se, current treatment methods for AKI can only slow down the progress of the condition. The management is mainly focused on supportive treatment by achieving and maintaining hemodynamic stability and avoiding hypovolemia to assure adequate renal perfusion [199]. When AKI has potentially life-threatening complications renal replacement therapy is required [94, 95]. Several pharmacological agents have developed on the basis of recent advances in renal injury and repair signaling pathways, such as recombinant alkaline phosphatase, p53 siRNA and levosimendan [98]. However, no pharmaceutical agents are successful in clinical trials so far. Given the complex pathogenesis of AKI and lack of treatment of AKI, it might be necessary to develop therapies that simultaneously targets multiple pathways [200].

siRNA has received increasing attention as a therapeutic approach for treating various diseases through silencing specific genes [201-203]. Unlike other gene
technologies, siRNAs have several unique advantages, such as high degree of specificity to mRNAs [204], non-immunogenic property [205] and high resistance to nucleases [206]. Several studies successfully ameliorate AKI by inhibiting gene expression with siRNA. Kim and co-workers in the same group used hydrodynamic injection method to inhibit Tp53-induced glycolysis and apoptosis regulator (TIGAR) expression. By doing so, they showed renoprotective effect on ischemic kidney injury [207]. However, the hydrodynamic injection produces high pressure during administration which will lead to liver damage and local renal dysfunction. Therefore, this method cannot be used in large animals and humans [208]. To safely and efficiently deliver siRNAs to kidneys, limited studies developed carrier systems and successfully attenuated AKI. Alidori and co-workers selectively delivered siRNAs targeting both p53 and Mep1b with ammonium-functionalized carbon nanotube (fCNT) to renal tubule cells in AKI and the renal injury was significantly ameliorated [136]. Yang et al. also prevented kidney damage by using chitosan/siRNA nanoparticles targeting cyclooxygenase type 2 (COX-2) [120]. Thus, using polymeric carrier systems to deliver siRNA in kidneys may be one of promising pathways for therapeutic strategy for AKI.

Cytokines and chemokines are elevated in response to AKI, leading to a proinflammatory microenvironment [157]. C-X-C chemokine receptor 4 (CXCR4) is one of the most commonly expressed chemokine receptors in many tissues, which plays a vital role in the process of inflammatory diseases or the metastasis in multiple types of cancers and [162-165]. Binding with its ligand, stromal cell-derived factor-1 (SDF-1), divergent intracellular signaling transduction pathways are initiated, which can result in a variety
of responses such as chemotaxis, cancer cell proliferation, recirculation and hematopoiesis of leukocytes [166, 167]. Emerging evidence shows that the increased chemokine receptor CXCR4 and its ligand SDF-1 are involved in the AKI pathology [164]. The CXCR4/SDF-1 axis is implicated in regulating trafficking and invasion of inflammatory cells in the injured kidneys. The inhibition of the axis appears to exert beneficial therapeutic effect in experimental AKI animal models [173, 175]. For example, Yuan et al, demonstrated that the mouse renal fibrosis was attenuated after administration of AMD3100 which is CXCR4 antagonist [175]. Therefore, interference the CXCR4/SDF-1 signaling pathway will be another promising therapeutic method for the treatment of AKI.

In this study, we report the development of a novel combinational therapeutic strategy for treatment of AKI based on previously synthesized dual-function polymeric CXCR4 inhibitors (PCX) which is also capable of systemically delivering siP53 to kidneys with AKI. Our previous study suggested that PCX was able to function in such a dual manner [209]. Here, we hypothesized that the PCX/siP53 polyplexes will lead to an improved therapeutic effect on AKI through dual pathways of antagonizing CXCR4 and down regulating p53.

2. Materials and Methods

2.1 Materials

Cyclam (1,4,8,11-tetraazacyclotetradecane) was obtained from Alfa Aesar (Ward Hill, MA). N,N'-hexamethylenebisacrylamide (HMBA) was from Polysciences, Inc. (Warrington, PA). Trifluoroacetic acid (TFA) was purchased from ACROS Organics (Fair Lawn, NJ). RPMI 1640 medium, Dulbecco’s phosphate buffered saline (PBS), and fetal
bovine serum (FBS) were from Thermo Scientific (Waltham, MA). Mouse
glyceraldehyde 3-phosphate dehydrogenase siRNA (siGAPDH sense, 5’-
CAGAAGACUGUGGAUGGCC (DTDT)-3’, antisense, 5’-
GGCCAUCCACAGUCUUCUG(DGDG)-3’) were purchased from GE Healthcare
Dharmacon, Inc. (Lafayette, CO). Cyanine 3 (Cy3) NHS ester and Cyanine 5.5 (Cy5.5) NHS
ester were from Lumiprobe (Hunt Valley, MD). Rotor-Gene SYBR Green RT-PCR Kit
was from QIAGEN (Hilden, Germany). TRIzol® reagent was purchased from Life
Technologies (Carlsbad, CA). Mouse CXCR4 primers (Forward, 5’-
CTGTGACCGCTTCTACCCCAATGACTT-3’, Reverse, 5’-CTTGGGGTAGGAGATACG
AAAGGAACC-3’) and GAPDH primers (Forward, 5’- ACGACCCCCTTCATTGAC-3’,
Reverse, 5’-TCCACG ACATACTCAGCAG-3’) were purchased from Sigma-Aldrich. The
normalized primers of phosphoglycerate kinase 1 (PGK1) (Forward, 5’-GCAGATTT
TGGAATGGTC-3’, Reverse, 5’-TGCTCACATGGGCTGACTTTA-3’) were from Real Time
Primers. All other reagents were from Fisher Scientific and used as received unless
otherwise noted.

2.2 Methods

2.2.1 Synthesis and Characterization of PCX Polymer

The process of synthesis and characterization of PCX followed our previous
published method [209].
2.2.2 Preparation and Characterization of PCX/siRNA Polyplexes

PCX/siRNA polyplexes were prepared in 10 mM HEPES buffer (pH 7.4) at predetermined w/w ratios by mixing equal volume of PCX and siRNA solutions. The polyplexes were allowed to form at room temperature for 20 min before use. Polyplexes prepared at different PCX-to-siRNA weight ratios were loaded (20 µL of the sample containing 1.0 µg of microRNA) and run for 30 min at 75 V in 0.5 × Tris/Borate/EDTA buffer. The gels were visualized under UV illumination on a KODAK Gel Logic 100 imaging system.

siRNA release from polyplexes was analyzed by heparin displacement assay. The polyplexes were prepared at a w/w ratio of 10 and incubated with increasing concentrations of heparin for 30 min at room temperature. The samples (20 µL of the sample containing 0.5 µg of siRNA) were then analyzed by agarose gel electrophoresis.

Hydrodynamic diameter and ζ-potential were determined by dynamic light scattering using a ZEN3600 Zsizer Nano-ZS (Malvern Instruments Ltd., Massachusetts, United States). Results were expressed as mean ± standard deviation (SD) of three experimental runs for each sample. The morphology of complexes was observed by transmission electron microscopy (TEM, Tecnai G2 Spirit, FEI Company, USA).

2.2.3 Cellular Uptake and Intracellular Trafficking of PCX/siRNA Polyplexes

Flow cytometry analysis was used to study the cellular uptake of polyplexes. MCT cells (10⁵) were seeded in 12-well plates. On the other day, the cells were incubated with
PCX/FITC-Oligo polyplexes at a FITC-Oligo concentration of 50 nM for 4 h. The cells were then trypsinized, washed with PBS and subjected to analysis using a BD FACSCalibur flow cytometer (BD Bioscience, Bedford, MA). The results were processed using FlowJo software.

Intracellular trafficking was observed by LSM 800 Laser Scanning Microscope (Zeiss, Jena, Germany). MCT cells were cultured on 24-well plates with round coverslip glass at 5 × 10^4 cells/well. After 24 h, a solution of polyplexes prepared at different w/w ratios was added at FITC-Oligo concentration of 50 nM. After incubation for another 4 h, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min and nuclei were stained with Hoechst 33258 for another 10 min.

### 2.2.4 Induction of Kidney Injury

To induce AKI, two different animal models were used, unilateral I/R injury and cisplatin-induced injury. All animal experiments followed a protocol approved by the UNMC Institutional Animal Care and Use Committee. For unilateral I/R injury, CD-1 male mice (8-10 week old) were purchased from Charles River. All animals were given free access to food and water. The non-fluorescent food was given at least 1 week before surgery. The mice were anesthetized by intraperitoneal administration of ketamine (200 mg/kg) and xylazine (16 mg/kg). Following left dorsal flank incision, renal pedicles were bluntly dissected and a microvascular clamp (Roboz Surgical Instrument, Gaithersburg, MD) was placed on the left renal pedicle for 35 min to induce unilateral I/R injury. During the procedure, mice were kept well hydrated with warm saline and on 37 °C heating pad.
After 35 min of occlusion, the clamps were removed and kidney reperfusion was verified visually. Then wound clips were used to close skin. Sham-operated right side underwent the same surgical procedure, except for the occlusion of the renal arteries. The mice were monitored until they woke up. Cisplatin nephrotoxic AKI was induced in CD-1 male mice (aged 8–10 weeks). Mice were intraperitoneally injected with a single dose of cisplatin at 15 mg/kg after starvation for 18 h.

2.2.5 In vivo Biodistribution of Polymers

Biodistribution of the polymers in mice with cisplatin-induced injury was analyzed by ex vivo fluorescence imaging. siRNA was labeled with Cy5.5 and PCX polymers were labeled with Cy3. Free PCX-Cy3, free siRNA-Cy5.5 or polyplexes were administered via tail vein injection 72 h after cisplatin administration. At 4 h and 24 h after administration of free PCX-Cy3, free siRNA-Cy5.5 or polyplexes, mice were sacrificed and major organs were isolated and imaged using Xenogen IVIS 200 at designated emission and excitation wavelength. The fluorescence intensity from kidney was quantified using Living Image® 4.5 software. The radiant efficiency of the kidney was measured as (photons/sec/cm²/sr)/(µW/cm²). Background fluorescence was subtracted prior to analysis.

To study intracellular localization of different polymers in kidney, bisected kidneys were embedded in OCT compound and cut into 10 µm frozen sections. The frozen sections were either stained with DAPI, or stained with H&E. The localizations of free siRNA-Cy5.5, free PCX-Cy3 and polyplexes were visualized by LSM 800 Laser Scanning Confocal Microscope (Zeiss, Jena, Germany). Histopathological change was confirmed by H&E sections under EVOS microscope.
2.2.6 Renal Function Deterioration

Blood was collected for BUN (QuantiChrom™ Urea Assay Kit, BioAssay Systems, USA) and creatinine (QuantiChrom™ Creatinine Assay Kit, BioAssay Systems, USA) measurements. Kidneys were dissected for paraffin embedding and for qRT-PCR. Paraffin sections (4 mm) were stained with periodic acid-Schiff (PAS) by tissue facility at UNMC. Histopathological change was confirmed by PAS sections under EVOS microscope.

2.2.7 RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR)

The expression mRNA levels of CXCR4 and GAPDH were evaluated by TaqMan qRT-PCR. Kidneys and other tissues were homogenized in TRIzol® reagent (Life Technologies, Carlsbad, CA) according to manufacturer’s protocol for total RNA extraction. 2 µg of total RNA was converted into cDNA using High Capacity cDNA Reverse Transcription Kits. The obtained cDNA then served as the template for qRT-PCR using the iTaq™ Universal SYBR Green kit (BIO-RAD) running on a Rotor-Gene Q instrument (QIAGEN) according to the manufacturer’s instructions. mRNA expression levels were expressed relative to the internal control according to the comparative threshold cycle (Ct) method. Primers used for qRT-PCR amplification are specified in Materials.

2.2.8 Statistical Analysis

Data are presented as the means ± SD. The statistical significance of the mean values of all groups was determined by using one-way ANOVA. An unpaired t test was used to
compare the means of two different groups. A $P$ value < 0.05 was considered statistically significant.

3. Results and Discussions

3.1 CXCR4 Expression in Kidneys with AKI

Chemokines are vital of importance in inflammation. They provide chemoattractive signals for leukocytes activation and migration [210]. The increase in expression of CXCR4 in the injured kidneys has been reported by several papers [164, 211]. Here, we showed renal CXCR4 mRNA and protein level in kidneys after unilateral I/R injury. We induced IR injury on left kidney (I/R kidney) while the right kidney received sham surgery (healthy kidney). At the designated time points after surgery, we conducted paraffin sections from healthy and IR kidneys to determine the CXCR4 protein level by immunohistochemistry analysis. The relative mRNA level was determined by qRT-PCR analysis followed by RNA extraction. For cisplatin-induced mouse model, similar procedures were conducted at 72 hours after cisplatin administration. The CXCR4 staining in healthy kidney showed undetectable expression (Figure 8A), while it showed a positive patchy pattern in I/R kidney and cisplatin injured kidney. Based on Figure 8A, we can see tubule cells are CXCR4 positive while glomerulus is CXCR4 negative. There is more intense staining in distal tubule compared to proximal tubule cells. H&E staining confirms the pathophysiology of healthy and injured kidneys. Figure 8B is the relative CXCR4 mRNA level in IR kidney and cisplatin injured kidney. For I/R kidney, CXCR4 mRNA level shows a gradient increase along with time passing. At 1 day after surgery, the CXCR4 mRNA
level in I/R kidney is 1.6 times as much as that in healthy kidney. At 13 days after surgery, the CXCR4 mRNA level in I/R kidney is about 4.2 times more than that in healthy kidney.

Based on the result of CXCR4 protein and relative mRNA level, we concluded that the CXCR4 expression increases in the kidney with unilateral I/R animal model. The increased CXCR4 in I/R kidney does not show obvious effect on the CXCR4 level in the contralateral healthy kidney.
Figure 8. Characterization of CXCR4 expression in kidney with AKI. (A) H&E staining and CXCR4 immunostaining for healthy and I/R kidney. (B) Relative CXCR4 mRNA level in I/R kidney. Data are shown as mean ± SD (n = 3).
3.2 Preparation and Characterization of PCX/siRNA Polyplexes

The ability of PCX to form polyplexes with siRNA was evaluated by agarose gel electrophoresis assay. In Figure 9A, there was a smear band at w/w ratio of 0.5 and 1. PCX can fully condense siRNA at a w/w ratio of 2 or above as indicated by no fluorescence signal coming out of the well. The ability of PCX/siRNA polyplexes to release siRNA was then evaluated by heparin displacement assay (Figure 9B). For PCX/siRNA polyplexes prepared at w/w 5, the siRNA was not fully released until the heparin concentration was 80 µg/mL, which means the polyplexes were able to release siRNA.

Hydrodynamic size and zeta-potential of PCX/siRNA polyplexes prepared at various w/w ratios were measured by dynamic light scattering (Figure 9 C&D). Polyplexes prepared at w/w 2 showed a relative large size with around 300 nm while the size decreased to ~127 nm at w/w 5 and ~107 nm at w/w 10. Polydispersity indexes (PDI) was less than 0.2 for all the three formulations. As expected, due to the redundant cationic PCX around the surface of polyplexes, increasing the w/w ratio to make polyplexes leads to the increased zeta-potential. The morphology of PCX/siRNA polyplexes was confirmed by transmission electron microscopy (TEM). Nice sphere shape of PCX/siRNA polyplexes prepared at all the three w/w ratios was observed under TEM (Figure 9E).
Figure 9. Characterization of polyplexes of PCX/siRNA. (A) siRNA condensation by PCX in 10 mM HEPES buffer (pH 7.4) using agarose gel electrophoresis. (B) Heparin induced siRNA release from the polyplexes. Polyplexes were prepared at w/w 5 and incubated with increasing concentrations of heparin. (C) DLS size distribution of PCX/siRNA. (D) Zeta-potential of PCX/siRNA. (E) TEM images of PCX/siRNA. Data are shown as mean ± SD (n = 3).
3.3 Intracellular localization and Cellular Uptake of PCX/siRNA polyplexes

To study the intracellular localization and cellular uptake, the siRNA used to form polyplexes with PCX was labeled with FAM fluorescence. MCT cells were treated with PCX/FAM-siRNA for 4 h followed by fixation for confocal microscopy. Cell nuclei were stained with Hoechst 33258 which was indicated by blue color. As shown in Figure 10A, for PCX/FAM-siRNA prepared at all the three w/w ratios, the most fluorescence of FAM-siRNA was distributed in cytoplasm while no fluorescence was observed in the cell nucleus.

Next, we investigated the cellular uptake of PCX/siRNA polyplexes prepared at various w/w ratios. MCT cells were incubated with the PCX/FAM-siRNA polyplexes for 4 h and cellular uptake was determined by flow cytometry. As shown in Figure 10B, the significantly increased mean fluorescence intensity (MFI) indicated that PCX polyplexes had significant cellular uptake in MCT cells when compared with untreated cells or cells treated with free FAM-siRNA. The cell uptake increased when the w/w ratios in preparation of the polyplexes increased from 2 to 10. The percentage of cells that took up the polyplexes (Figure 10C) showed similar trend as MFI data. PCX/siRNA polyplexes prepared at w/w ratio of 10 were selected for subsequent in vivo study as their highest cell uptake.
Figure 10. Intracellular trafficking and cellular uptake of PCX/siRNA polyplexes. (A) Intracellular trafficking of PCX/FITC-siRNA in MCT cells by confocal after 4 h of incubation. Quantification of cellular uptake is shown by (B) mean fluorescence intensity (MFI) and (C) % cell uptake. Data are shown as mean ± SD (n = 3).
3.4 Biodistribution of PCX/siRNA polyplexes in vivo

Accumulation of polyplexes in focus is the prerequisite for therapeutic treatment. Before measuring therapeutic effect of PCX/siRNA polyplexes, it is necessary to study the biodistribution of the polyplexes in AKI mouse model.

To understand whether PCX/siRNA polyplexes can target injured kidneys, the biodistribution of polyplexes was conducted. The mouse model we used to study biodistribution is well-established mouse model, cisplatin-induced nephrotoxic injury. To track polyplexes, we labeled PCX with Cy3 and siRNA with Cy5.5. Mice were sacrificed at two designated time points, 4 h or 24 h post intravenously administration and major organs were isolated for ex vivo fluorescence imaging by IVIS. As shown in Figure 11A, a slightly more fluorescence of free siRNA was in healthy kidneys at 4 h after administration and most siRNA was excreted from kidneys at 24 h after injection. On the contrary, significant fluorescence of siRNA of polyplexes was observed in injured kidneys compared with healthy kidneys. No obvious fluorescence was shown in other organs except for the weak signal in liver and spleen. Quantification of fluorescence of siRNA was shown in Figure 11B. For polyplexes, the fluorescence intensity increased approximately by 50% in injured kidneys compared with healthy kidneys at 4 h post injection. However, there was no difference between healthy and injured kidneys after free siRNA administration.

As shown in Figure 11C, the PCX showed potential to accumulate in injured kidneys for both free PCX and polyplexes. At 24 h, most PCX of polyplexes was excreted while
free PCX remained in injured kidneys. No obvious fluorescence was shown in other organs except for the weak signal in liver and spleen. Fluorescence quantification showed that, at 4 h post injection, the fluorescence intensity of PCX of polyplexes in injured kidneys was ~7 times as much as that in healthy kidney (Figure 11D).

Given the complex structure of kidney, we were further interested in suborgan distribution of the polyplexes. To investigate the intracellular localization of PCX/siRNA polyplexes, frozen kidney sections were visualized under confocal microscopy. As shown in Figure 12A, most polyplexes cross glomerulus filtration barrier and accumulate in injured mouse tubule cells at 4 h after intravenous administration. Enlarged fenestration of filtration barrier in injured kidney may allow large polyplexes to pass through this barrier. An alternative possibility is that polyplexes could disassemble at glomerular basement membrane and a fraction of PCX reassociate with siRNA after filtrating into urinary space and facilitate uptake of siRNA in tubule cells [114]. The yellow dots in merged images indicated intact polyplexes. The ability of renal tubule cells to uptake systemically injected siRNA has been well-known and extensively used [212, 213]. As expected, free siRNA passed glomerulus filtration barrier and accumulated in mouse tubule cells of both healthy and injured kidney at 4 h after intravenous administration (Figure 12B). The intracellular localization of free PCX in kidneys was evaluated as well (Figure 12C). Consistent with IVIS result, a lot of PCX accumulated in injured kidney while few PCX accumulated in healthy kidney. In injured kidney, large amount of PCX crossed glomerulus filtration barrier and distributed in tubule cells other than glomeruli which is indicated by white arrow.
Based on above results, we confirmed that PCX/siRNA polyplexes are able to specifically target renal tubule cells in cisplatin-induced nephrotoxic mouse model, which prompted us to further investigate the in vivo transfection efficacy of PCX/siRNA.
**Figure 11.** Biodistribution of PCX/siRNA polyplexes in healthy mice and cisplatin-injured mice. Ex vivo imaging of dissected organs at 4 or 24 h after administration of free siRNA (A) and polyplexes (B). Excitation wavelength is 675 nm and emission wavelength is 720 nm. Organs include heart, lung, liver, spleen and kidneys (from left to right and top to bottom). Quantification of fluorescence of siRNA-Cy5.5 from free siRNA (C) and polyplexes (D) was shown.
Continued Figure 11. Biodistribution of PCX/siRNA polyplexes in healthy mice and cisplatin-injured mice. Ex vivo imaging of dissected organs at 4 or 24 h after administration of free siRNA (A) and polyplexes (B). Excitation wavelength is 535 nm and emission wavelength is 580 nm. Organs include heart, lung, liver, spleen and kidneys (from left to right and top to bottom). Quantification of fluorescence of PCX-Cy3 from free PCX (C) and polyplexes (D) was shown.
Figure 12. Confocal microscopy images of kidney sections. Kidneys were extracted from mice with healthy and cisplatin-induced injury at 4 h after receiving polyplexes (A), free siRNA (B) and free PCX (C). Glomeruli is indicated by white arrow. Magnification, 20X.
3.5 In vivo transfection effect of PCX/siRNA polyplexes

Silencing specific gene expression in kidneys by siRNA through systemic hydrodynamic injection is a common method for siRNA delivery in rodents [207, 214]. Hydrodynamic injection via systemic circulation can cause transient high pressures in veins during rapid injection of a large volume, leading to enhanced delivery of siRNA into organs with high blood flow, such as kidneys and liver [215, 216]. Although this method has been proven useful for a wide range of application in rodents, it is not feasible in humans due to the possible cardiac congestion [217]. Based on above biodistribution result of PCX/siRNA polyplexes, therefore, we will further measure silencing efficacy of the polyplexes in vivo. We chose glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as target gene due to its stable expression in the body. Procedure of experiment is shown in Figure 13A. 1.67 mg/kg (50 µg) siGAPDH dose was used for hydrodynamic injection. 1.12 mg/kg (0.28 mg/kg × 4, free siGAPDH and w/w 5) and 0.56 mg/kg (0.14 mg/kg × 4, w/w 10) siGAPDH doses were used for polyplex injections. To avoid individual difference and reduce the risk of mortality of mice, we established a unilateral IRI model, where left renal artery was clamped and renal injury was induced in the left kidney while the right kidney was also exposed and remained intact. After treatment, as shown in Figure 13B, there was approximately 25% down regulation of GAPDH mRNA in injured kidneys treated with PCX/siGAPDH prepared at w/w 5. The silencing efficacy of PCX/siGAPDH was slightly better than hydrodynamic injection, but the siGAPDH dose of PCX/siGAPDH was only two thirds of the dose of hydrodynamic injection. One study reported there was about 40% of GAPDH silencing in kidneys after hydrodynamic
injection with an approximate siGAPDH dose of 7.5 mg/kg and other organs such as spleen and liver also showed detectable GAPDH silencing [218]. There was no obvious silencing for free siGAPDH although its dose was same to polyplexes prepared at w/w 5. For polyplexes prepared at w/w 10, only about 18% down regulation of GAPDH mRNA level due to less injected siGAPDH amount.

In summary, the polyplexes of PCX/siGAPDH prepared at w/w 5 showed a significant GAPDH silencing in targeted injured kidney at an extremely low siGAPDH amount. This finding is promising for further therapeutic study of polyplexes in AKI.
Figure 13. Silencing efficacy of PCX/siGAPDH polyplexes in unilateral ischemia-reperfusion mouse model. Experiment was conducted followed the procedure in (A). The relative GAPDH mRNA level was measured after treatment (B). Data are shown as mean ± SD (n = 3)
3.6 *In vivo* Therapeutic Effect of PCX/siRNA Polyplexes

Results from the distribution study of PCX/siRNA complexes gave us enough confidence to proceed to investigate the therapeutic efficacy of our formulations as a preconditioning therapy for AKI. First, we evaluated the therapeutic efficacy of PCX only in the cisplatin-induced animal model. As shown in Figure 14A, mice were prophylactically treated for four consecutive days with PCX, AMD3100 or PBS. On the third day, mice received a single nephrotoxic dose of cisplatin to induce AKI (15 mg/kg). Blood was collected on day 4 and day 5 for renal function study. Then mice were sacrificed on day 5 and kidneys were collected for PAS staining of paraffin sections. Based on the level of BUN and Creatinine in Figure 14 B&C, AMD3100 and our PCX polyplexes didn’t show any obvious nephrotoxicity in healthy mice. For mice induced AKI by cisplatin, the BUN and Creatinine level significantly reduced after the treatment of AMD3100 and PCX, which indicated that antagonism of CXCR4 has renoprotective effect on AKI. However, the renal protective effect was similar between AMD3100 and our PCX polymers.

Histological analysis of kidneys from those mice was performed. PAS stained kidneys from healthy mice receiving AMD3100 or PCX showed normal tissue morphology. The morphology of kidneys from injured mice receiving AMD3100 or PCX also showed consistence with healthy control mouse tissue (Figure 15A). The percentage of damaged tubules were quantified and shown in Figure 15B.
Figure 14. Therapeutic effect of PCX. Animal experiment was conducted in cisplatin-induced injury model. The timeline was shown in (A). Renal function deterioration was evaluated by BUN (B) and serum creatinine (C).
Figure 15. Histological evaluation of the effect of PCX on tubule damage in response to cisplatin-induced AKI. (A) Representative images of PAS staining of kidney sections (20X). (B) PAS analysis of renal tubule damage (PBS, n = 3; AMD3100, n = 4; PCX, n = 5)
4. Conclusion

In summary, PCX/siRNA could be specifically delivered into injured kidney following intravenous injection, where it can persist for about 24 h. Treatment with PCX can mitigate AKI. And injections of very low doses of GAPDH siRNA showed similar silencing effect to hydrodynamic injection. These data presented here provide a potential generic platform of gene therapy for treatment of AKI and may further augment the potential clinical application of siRNA for AKI. In the future, the pathophysiological roles of this novel pharmacological approach will be evaluated in several proteins overexpressed during AKI.
Chapter 4. Conjugate polyplexes with anti-invasive properties and improved siRNA delivery in vivo

1. Introduction

siRNA has received increasing attention as a therapeutic approach for treating various diseases through silencing specific genes [201-203]. The use of naked unformulated siRNA, however, is associated with several inherent problems due to the poor cellular permeability, easy degradation by endogenous enzymes and off-target effects [149]. To efficiently deliver siRNAs to the target tissues in vivo, a wide variety of non-viral carrier systems, such as cationic lipids and cationic polymers, have been developed to form polyelectrolyte complexes with the oppositely charged siRNA [150, 151]. These cationic carriers provide high transfection efficiency in vitro but suffer from low activity in vivo. The cationic carriers may not form sufficiently strong interactions with anionic siRNA simply through electrostatic interactions because of the relatively small size of siRNA [219, 220]. Polycations have a tendency to interact with multiple proteins in serum and cause aggregation which can lead to fatal disseminated intravascular coagulation-like condition [221-223]. In addition, because of their dynamic nature, polyplexes are susceptible to disassembly by various competing biomacromolecules in the systemic circulation or at a glomerular basement membrane in the kidneys by the action of heparan sulfate [224-230]. Many solutions have been proposed to the premature systemic disassembly, including modifications with hydrophobic residues and covalent crosslinking of the particles [224, 231-233]. To overcome the limitations of siRNA in effective electrostatic condensation with polycations, polymerized siRNA was used and
shown to form stable polyplexes with efficient intracellular translocation and targeted
gene silencing in vitro [234, 235]. Finally, direct covalent linkage of siRNA to synthetic
polymers can significantly improve the performance during systemic siRNA delivery
[236-238].

Human serum albumin (HSA) is the most abundant protein in human body. HSA is
involved in many important biological functions, such as delivery of nutrients from
circulating system to cells and maintenance of osmotic pressure and vascular integrity. As
a natural transport protein, HSA is able to bind with various endogenous and exogenous
molecules [239, 240]. In addition, its target specificity for glycoprotein 60 receptor, which
is presented on the surface of cancer cells, allows potential site-specific delivery of various
anti-cancer agents. Several studies also showed that albumin-based drug delivery systems
can accumulate in solid tumors and facilitate tumor targeting due to passive targeting by
enhanced permeability and retention (EPR) effect [241, 242]. Albumin has been also
explored in improving the safety and efficacy of polyplexes. Because of its negative net
charge, albumin has been utilized to coat polyplexes with the goal to avoid hepatic
accumulation, reduce toxicity and improve delivery of therapeutic nucleic acids [240, 243].
PEI/DNA polyplexes coated with albumin showed improved gene transfer efficiency with
negligible toxicity [244-246]. Stable ternary polyplexes were developed by modification of
PEI with ligands for albumin binding [247]. Interestingly, even unmodified HSA
improved the internalization and silencing efficiency of PEI/siRNA polyplexes in cancer
cells [248]. Unfortunately, very few studies report on the use of albumin-coated polyplexes in vivo.

CXCR4 is one of the most commonly expressed chemokine receptors, which plays a vital role in the process of metastasis in multiple types of cancer [249]. Binding with its ligand, stromal cell-derived factor-1 (SDF-1), divergent intracellular signaling transduction pathways are initiated, which can result in a variety of responses such as chemotaxis, cancer cell proliferation, migration and invasion [166]. Driven by CXCR4 expressed in the primary tumor cells, the cells metastasize to secondary sites where SDF-1 is highly expressed, such as lung, liver, bone marrow and brain [250]. Blocking the CXCR4/SDF-1 interaction by antagonizing CXCR4 can inhibit macrophage infiltration, induce tumor growth arrest and prevent metastatic spread [165, 172]. We previously reported the synthesis of hyperbranched bioreducible polycationic CXCR4 inhibitors named rPAMD [251-254]. The rPAMD had the ability to inhibit cancer cell invasion as a result of their CXCR4 inhibitory activity, while mediating efficient transfection in vitro. To further advance development of rPAMD for systemic delivery of siRNA, we hypothesized that conjugating thiol-modified siRNA to rPAMD will enhance stability against disassembly and that coating of such conjugates with HSA will decrease the positive surface charge and enhance safety of the formulations (Scheme 1). Here, we have tested a simple thiol-disulfide exchange conjugation strategy to prepare rPAMD-SS-siRNA polyplex conjugates and tested their stability in serum and against heparin exchange. Reporter gene silencing activity of the polyplex conjugates was tested in B16F10-luc
mouse melanoma cells to determine how the covalent conjugation and HSA coating affects the silencing efficacy. Finally, the systemic siRNA delivery efficacy of the developed polyplex conjugates was tested in vivo in a syngeneic tumor model.

2. Materials and Methods

2.1 Materials

AMD3100 base form was purchased from Biochempartner (China). N,N’-cystamine-bisacrylamide (CBA) was obtained from Polysciences, Inc. (Warrington, PA). Ethidium bromide (EtBr) was from Fisher Bioreagents (Fair Lawn, N.J.). Dithiothreitol (DTT) was obtained from Alfa Aesar (Heysham, LA3 2XY, England). Scrambled siRNA (siScr, sense: 5’-AUG AAC GUG AAU UGC UCA AUU-3’), luciferase siRNA (siLuc, sense: 5’-GGA CGA GGA CGA GCA CUU CUU-3’), thiol-modified scrambled siRNA (siScr-SH, sense: 5’-S-S-AUG AAC GUG AAU UGC UCA AUU-3’), thiol-modified luciferase siRNA (siLuc-SH, sense: 5’-S-S-GGA GGA CGA GGA GCA CUU CUU-3’) were custom-synthesized by Dharmacon, GE, and deprotected by tris(2-carboxyethyl) phosphine hydrochloride (TCEP) solution following the manufacturer’s instructions. Dulbecco’s Modified Eagle Medium (DMEM), RPMI-1640 Medium, Dulbecco’s Phosphate Buffered Saline (PBS), and Fetal Bovine Serum (FBS) were from Thermo Scientific (Waltham, MA). Cell culture inserts for 24-well plates with 8.0 μm pores (Translucent PET Membrane, cat# 353097) and BD Matrigel™ Basement Membrane Matrix (cat# 354234) were obtain from BD Biosciences (Billerica, MA). Human SDF-1 was from Shenandoah Biotechnology, Inc. (Warwick, PA). All other reagents were from Fisher Scientific and used as received unless otherwise noted.
2.2 Methods

2.2.1 Synthesis and Characterization of rPAMD

rPAMD was synthesized through Michael-type polyaddition with equal molar ratio of AMD3100 and a reducible bisacrylamide CBA [253]. Briefly, AMD3100 (100.4 mg, 0.4 mmol) and CBA (52 mg, 0.4 mmol) were dissolved in methanol/water mixture (2 mL, 7/3 v/v). The reaction was stirred under nitrogen at room temperature for 48 h. Excess AMD3100 (10 mg) was added to react with any residual CBA acrylamide groups. After 6 h of continuous stirring, 1.25 M HCl was added dropwise to the mixture until the pH reached 4. The resulting HCl salt of rPAMD was collected and dialyzed (molecular weight cut-off 3.5 kDa) against acidified water (pH 4) for 3 days before final lyophilization. \(^1\)H-NMR was used to confirm the chemical composition of the synthesized rPAMD and AMD3100 content in the polymers. The completion of the reaction was validated by the disappearance of the acrylamide peak of CBA. The weight- and number-average molecular weights, as well as the polydispersity index (PDI, \(M_w/M_n\)) was characterized by gel permeation chromatography (GPC) operated in 0.1 M sodium acetate buffer (pH 5.0) using Agilent 1260 Infinity LC system equipped with a miniDAWN TREOS multi-angle light scattering (MALS) detector and a Optilab T-rEX refractive index detector (Wyatt Technology, Santa Barbara, CA). The column used was Tosoh Bioscience TSKgel G3000PWXL-CP eluted at a flow rate of 0.5 mL/min. Results were analyzed using Astra 6.1 software from Wyatt Technology.
2.2.2 Preparation and Characterization of HSA-coated Polyplexes

rPAMD/siRNA polyplexes were prepared in 5 mM HEPES buffer (pH 7.4) at predetermined w/w ratios by mixing equal volume of rPAMD and siRNA solutions. The polyplexes were allowed to form at room temperature for 20 min before use. Similarly, rPAMD-SS-siRNA conjugate polyplexes were prepared by mixing of rPAMD and siRNA-SH in 5 mM HEPES buffer (pH 7.4) and kept at room temperature for 2 h before use. To prepare HSA-coated polyplexes, which are named as HSA[rPAMD/siRNA] and HSA[rPAMD-SS-siRNA], HSA solution in 5 mM HEPES buffer (pH 7.4) was added to the prepared polyplexes or conjugate polyplexes at a HSA/rPAMD w/w ratio of 5. The mixture was allowed to stand at room temperature for 20 min before use. Hydrodynamic diameter and ζ-potential were determined by dynamic light scattering using a ZEN3600 Zsizer Nano-ZS (Malvern Instruments Ltd., Massachusetts, United States). Results were expressed as mean ± standard deviation (SD) of three experimental runs for each sample.

2.2.3 Agarose Gel Electrophoresis

Polyplexes were prepared as above and incubated with increasing concentrations of heparin with or without 10 mM DTT. 18 µL of each sample (siRNA concentration: 20 µg/mL) were loaded onto a 2.5% agarose gel containing 0.5 µg/mL EtBr and run for 30 min under electrophoresis at 75 V in 0.5X Tris/Borate/EDTA (TBE) running buffer. Free siRNA of the same concentration was used as the control. The gel was then visualized under UV.
2.2.4 Cell Culture

Human epithelial osteosarcoma U2OS cells that stably express functional EGFP-CXCR4 fusion protein were obtained from Fisher Scientific and cultured in DMEM supplemented with 2 mM L-glutamine, 1% Pen-Strep, 0.5 mg/mL G418 and 10% FBS. Mouse melanoma cell line B16F10 expressing luciferase (B16F10-luc) was purchased from PerkinElmer and maintained in RPMI with 10% FBS.

2.2.5 Cytotoxicity

Cytotoxicity of the polyplexes and conjugate polyplexes was determined by Cell Titer Blue assay. The cells were plated in 96-well plates at a density of 10,000 cells/well (B16F10-luc) and 8,000 cells/well (U2OS). The next day, the culture medium was removed and replaced by 150 µL of serum-free medium containing polyplexes or conjugate polyplexes prepared at w/w 2 and 5 (rPAMD concentration was 2, 8 and 15 µg/mL for B16F10-luc cells and 2 µg/mL for U2OS cells). For B16F10-luc, the medium was replaced with fresh growth medium after 4 h incubation and the cells were cultured for another 48 h. For U2OS, the cells were incubated with polyplexes or conjugate polyplexes for 16 h. Then, the medium in each well was removed and a mixture of 100 µL serum-free media and 20 µL of CellTiter-Blue reagent (CellTiter 96®Aqueous Non-Radioactive Cell Proliferation Assay, Promega) was added. After 1 h incubation, the fluorescence (F) was measured using Synergy 2 Microplate Reader (BioTek, VT) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The relative cell viability (%) was calculated as \( \frac{[F}_{\text{sample}}}{[F]_{\text{untreated}}} \times 100\% \).
2.2.6 CXCR4 Redistribution Assay

U2OS cells overexpressing EGFP-CXCR4 were plated in black 96-well plates with optical bottom 24 h before experiment at a density of 8000 cells per well. On the day of the assay, cells were washed twice with 100 µL assay buffer (DMEM supplemented with 2 mM L-Glutamine, 1% FBS, 1% Pen-Strep and 10 mM HEPES). Then different formulations (2.0 µg/mL of polymer) or AMD3100 (300 nM) were added in the assay buffer containing 0.25% DMSO and incubated with cells for 30 min at 37°C. SDF-1 was then added to each well to make the final concentration 10 nM and cells were incubated at 37°C for another 1 h. After incubation, 4% formaldehyde at room temperature for 20 min, followed by 4 times of PBS washing and staining with 1 µM Hoechst 33258 for 30 min. Fluorescent microscopy was conducted using EVOS fluorescence microscope (20x). High content analysis was used to quantify the internalization of CXCR4 receptors using Cellomics ArrayScan VTI Reader and SpotDetector V3 BioApplication software. CXCR4 inhibitory activity of 300 nM AMD3100 was considered as positive control and set as 100% and cells treated with SDF-1 only was set as 0%. CXCR4 activity of all the tested particles was expressed as mean % inhibition ± SD (n = 3).

2.2.7 Cell Invasion Assay

Each transwell insert was coated with 40 µL diluted ice-cold Matrigel (1:3 v/v with serum-free medium) and placed in 37°C incubator for 2 h prior to experiment. CXCR4+ U2OS cells were trypsinized and resuspended in serum-free medium containing AMD3100 (300 nM), rPAMD/siRNA, HSA[rPAMD/siRNA], rPAMD-SS-siRNA or
HSA[rPAMD-SS-siRNA] (rPAMD 2.0 µg/mL). 100,000 treated cells in 300 µL medium were added to each insert, and 20 nM SDF-1 in serum-free medium was added to the corresponding wells in the companion plate as the chemoattractant. Cells were allowed to invade through the Matrigel layer towards SDF-1 at 37°C for about 18 h. The non-invaded cells were then removed using a cotton swab, and the invaded cells at the bottom of the insert were fixed with 100% methanol and stained with 0.2% Crystal Violet for 10 min. The images were taken by EVOS xl microscope (20x) and the number of invaded cells counted in triplicate.

2.2.8 siRNA Transfection in vitro

All transfection experiments were conducted in 48-well plates using cells at their logarithmic growth phase. B16F10-luc cells were seeded at a density of 8,000 cells/well 24 h prior to transfection. Different formulations with 100 nM siRNA were prepared as mentioned above in 250 µL serum-free medium and then added onto cells. After 4 h incubation, the medium was removed and replaced with fresh culture medium. Cells were cultured for another 48 h before measuring luciferase expression. For harvesting cells, 100 µL of 0.5x cell culture lysis buffer (Promega, Madison, WI) was added to the cells after discarding the culture medium and then lysed for at least 20 min at room temperature. The cell lysate was then transferred to 1.5 mL tubes and all the samples were centrifuged at 10,000 g for 10 min at 4°C. To measure the luciferase expression, 100 µL of 0.5 mM luciferin solution was automatically injected into each well containing 20 µL of cell lysate supernatant and the luminescence was integrated over 10 s using GloMax 96
Microplate Luminometer (Promega). Total cellular protein in the cell lysate was measured by the bicinchoninic acid protein assay using calibration curve constructed with standard bovine serum albumin solutions (Pierce, Rockford, IL). Transfection activity was expressed as % luciferase activity of siScr treated groups ± SD of triplicate samples.

2.2.9 siRNA Transfection in vivo

All animal experiments were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center. Six-week-old female C57BL/6 albino mice were subcutaneously injected with one million B16F10-luc cells and randomized to different treatment groups to avoid cage effects. When a tumor reached a volume of 50 mm³, the mice (in groups of 7) were injected intravenously (i.v.) with different polyplexes or conjugate polyplexes at siRNA dose of 1 mg/kg body weight daily for 4 days. Dextrose was added into each formulation to make a concentration of 5% before injection. The luminescence signal was detected in tumor before and after treatment using the Perkin Elmer IVIS bioluminescence imaging system. 24 h after the last injection, all mice were sacrificed, and the tumors were harvested and homogenized in 1x Cell Culture Lysis Reagent (Promega, Madison, WI) followed by centrifugation at 10,000 g for 5 min. The luciferase expression was measured as described in 2.7. Transfection activity was expressed as % luciferase expression relative to scrambled siRNA (siScr) control groups ± SD.
2.2.10 Statistical Analysis

Data are presented as the means ± SD. The statistical significance of the mean values of all groups was determined by using one-way ANOVA. An unpaired t test was used to compare the means of two different groups. A P value < 0.05 was considered statistically significant.

3. Results and Discussions

3.1 Preparation of Albumin-coated Polyplexes and Conjugate Polyplexes

Conventional siRNA polyplexes are formed by electrostatic interactions between polycations and the nucleic acid. One of the major challenges of these polyplexes when applied in vivo is their stability against disassembly with competing polyelectrolytes and other charged molecules in serum. Several viable solutions have been developed, including covalent conjugation of siRNA to polymer carriers [237]. As part of our long-term goal to develop CXCR4-inhibiting polycations (rPAMD) for siRNA delivery, we have adopted siRNA conjugation as a strategy to enable systemic administration of rPAMD/siRNA polyplexes. We proposed that assembling the hyperbranched bioreducible rPAMD with 5'-thiol functionalized siRNA will lead to facile in situ stabilization of the formed conjugate polyplexes by thiol-disulfide exchange reaction between siRNA-SH and the abundant disulfides in rPAMD (Scheme 3). We proposed that electrostatic interactions would dominate the initial formation of the polyplexes and that the thiol-disulfide exchange will then proceed rapidly within the formed polyplexes because of the local high concentration of the disulfides and thiols within the polyplexes (Scheme 3).
We have prepared both conventional polyplexes (rPAMD/siRNA) and conjugate polyplexes (rPAMD-SS-siRNA) at two different polymer/siRNA w/w ratios (2 and 5). The formation of the conjugate polyplexes was confirmed by assessing stability against polyelectrolyte exchange with heparin (Figure 16a). While no free siRNA was observed at any samples in the absence of heparin, the presence of as little as 60 µg/mL heparin caused complete dissociation of the conventional polyplexes prepared at w/w 2 and in the presence of 100 µg/mL heparin in case of polyplexes prepared at w/w 5. In contrast, the conjugate polyplexes showed strong resistance to heparin-induced disassembly at both w/w ratios, with only a small amount of free siRNA released by the action of heparin. The heparin exchange assay distinguishes between electrostatically and covalently bound siRNA. The small amount of free siRNA released from the conjugate polyplexes suggested that not all siRNA-SH participated in the thiol-disulfide exchange, most likely because of partial siRNA-SH oxidation. It is worth noting that the thiol-disulfide exchange may lead to a degradation of rPAMD. The extent of the disulfide cleavage, however, depends on the ratio of the siRNA-SH thiols to rPAMD disulfides present in the formulation. For example, formulations prepared at w/w ratio of 5 contain ~89 disulfides for each siRNA-SH. We assumed that the molecular weight of siRNA-SH is about 13 600 g/mol and that the molecular weight of the repeating unit per 1 disulfide in rPAMD is 764 g/mol. For a typical rPAMD/siRNA-SH formulation prepared at w/w ratio of 5, that translates to about 89 disulfides per each thiol (5/764)/(1/13 600). In such a case, the extent of rPAMD cleavage is negligible since only about 1% of the disulfides can be cleaved by the HS-siRNA.
Successful use of any polyplex stabilization strategy requires that the stabilization is reversible, and that siRNA is released in the cytoplasm. We have incubated the polyplexes with 10 mM DTT to simulate intracellular reducing environment and to confirm reversibility of the conjugate polyplexes. As shown in Figure 16a, disulfide reduction with DTT destabilized both the polyplexes and conjugate polyplexes and resulted in a complete release of siRNA. No differences were observed between polyplexes prepared at the different w/w ratios.

The effect of physiological salt concentration on the integrity of the polyplexes was investigated by incubation in PBS. High salt concentrations can weaken the electrostatic interactions between siRNA and polycation and cause polyplex disassembly. The destabilizing effect of PBS was clearly demonstrated in rPAMD/siRNA polyplexes by the significant amount of released siRNA over the 24 h incubation period (Figure 16b). Polyplexes prepared at w/w 5 were more resistant to salt dissociation than those prepared at w/w 2. As above, the conjugate polyplexes displayed greatly enhanced stability in physiological saline as documented by no significant siRNA release. Combination of high ionic strength with the complex mix of proteins and other charged molecules found in serum can further destabilize the polyplexes and so the stability was further tested in 50% serum (FBS) (Figure 16b). As expected, conventional polyplexes disassembled rapidly in the presence of serum and significant amount of the siRNA was degraded within 24 h as suggested by the fading band of the free siRNA in the gel. Conjugate polyplexes showed high stability against disassembly, although the appearance of fluorescence in the start
well of the conjugate polyplexes at w/w 2 suggested that serum at least partially disrupted the polyplexes structure and allowed binding of ethidium bromide to siRNA.
Scheme 3. Preparation of HSA[rPAMD-SS-siRNA] conjugate polyplexes
Figure 16. Stability of polyplexes and conjugate polyplexes against disassembly. (a) Heparin-induced siRNA release from the polyplexes and conjugate polyplexes prepared at w/w ratio = 2 and 5 in 5 mM HEPES buffer (pH 7.4) and incubated with increasing concentrations of heparin with (+) or without (-) 20 mM DTT. (b) Stability of the polyplexes and conjugate polyplexes in 50% FBS and PBS following incubation for 0-24 h. The release of free siRNA in both (a) and (b) was visualized by agarose gel electrophoresis. (c) Free siRNA degradation profile in 50% FBS.
3.2 Colloidal Characterization

Hydrodynamic size and zeta potential are important parameters that determine the cellular uptake, pharmacokinetics and biodistribution of polyplexes. We have measured the size and $\zeta$ potential of polyplexes and conjugate polyplexes prepared in 5 mM HEPES (pH 7.4) at both w/w ratios by dynamic light scattering (Figure 16 and 17). Polyplexes and conjugate polyplexes with both tested w/w ratios showed similar sizes in a range from 120-160 nm (Figure 16a) and positive $\zeta$ potentials ranging from 15 to 22 mV (Figure 17). The size of the conjugate polyplexes was slightly larger than the sizes of the conventional polyplexes. We have then assessed colloidal stability of both formulations by measuring the changes in size following a 4 h incubation in PBS (Figure 16b). As expected, the rPAMD/siRNA polyplexes showed poor colloidal stability indicated by rapid aggregation with sizes reaching nearly 800 nm within 4 h. In contrast, the conjugate polyplexes remained relatively stable even in PBS as their sizes increased only to 210-246 nm. When combined with the data in Figure 16b, the improvement in colloidal stability may suggest intra-particle crosslinking of the conjugate polyplexes by the catalytic action of the free thiols in siRNA-SH, similar to previously reported increase in colloidal stability of crosslinked DNA polyplexes [255].

3.3 Effect of albumin coating on polyplex properties

Both polyplex and conjugate polyplex showed highly positive surface charge, which made them ill-suited for in vivo use. Covalent conjugation of poly(ethylene glycol) (PEG) is the most common method to shield the positive surface charge of polyplexes. Here, we explored alternative approach to shielding the positive surface charge by adsorption of
HSA. Polyplexes and conjugate polyplexes were prepared as described above and then, HSA was added and allowed to adsorb to the surface of the polyplexes (Scheme 3). We first confirmed that HSA has no effect on polyplex stability against disassembly with heparin, serum, or PBS (Figure 16). The polyplexes coated with HSA showed slightly smaller hydrodynamic size in 10 mM HEPES (pH 7.4) (Figure 17a) but no effect of HSA on colloidal stability in PBS was observed (Figure 17b). Importantly, adsorption of the negatively charged HSA significantly decreased the ζ potential of both polyplexes and conjugate polyplexes in solution with pH 7.4 (Figure 18), thus making them better suited for the subsequent in vivo testing.

The HSA coating is strongly dependent on pH and because of the isoelectric point of albumin (4.9), we predicted that the coating would be reversed during endo/lysosomal trafficking of the polyplexes. We have thus prepared the coated polyplexes at pH 7.4 and then decreased the pH to 5 and observed the effect of the pH change on particle size (Figure 16c) and ζ potential (Figure 18). While the pH change had no significant effect of the particle size, we have observed significant increase in the ζ potential for both polyplexes and conjugate polyplexes. The ζ potential increased to the levels comparable to those found for the non-coated polyplexes, thus strongly suggesting that HSA dissociated from the polyplexes. Such behavior may be beneficial for the intracellular trafficking of the polyplexes as it may lead to exposure of unmodified rPAMD/siRNA in the endo/lysosomes and ultimately improve cytoplasmic transport.
Figure 17. Hydrodynamic size. Polyplexes and conjugate polyplexes were prepared at w/w ratio = 2 and 5 in 5 mM HEPES buffer (pH 7.4) and coated with HSA. Hydrodynamic size was measured by dynamic light scattering. (a) Hydrodynamic size in 5 mM HEPES buffer (pH 7.4); (b) Hydrodynamic size after 4 h incubation in PBS, and (c) Hydrodynamic size after adjustment of pH from 7.4 to 5. All data shown as mean ± SD (n = 3).
Figure 18. Zeta potential. Polyplexes and conjugate polyplexes were prepared at w/w ratio (a) 2 and (b) 5 in 5 mM HEPES buffer (pH 7.4), coated with HSA, and pH was adjusted to 5. All data shown as mean ± SD (n = 3).
3.4 Cytotoxicity and Transfection Activity in vitro

Another important consideration for systemic siRNA delivery is to minimize toxicity of the polyplexes. The use of bioreducible polycations such as rPAMD substantially decreases cytotoxicity when compared with non-reducible polycations [256-258]. To ensure that the formation of conjugate polyplexes and HSA coating had no reverse effects on cytotoxicity, we evaluated the effect of polyplexes and conjugate polyplexes on cell viability of a mouse melanoma B16F10-luc cell line (Figure 19). We have determined cell viability at three different rPAMD concentrations: 2, 8 and 15 µg/mL and found no significant adverse effect with any of the formulations as the cell viability remained above 90%.

The ability of the polyplexes and conjugate polyplexes to silence expression of the luciferase reporter gene was studied in B16F10-luc cells (Figure 20). Oligofectamine/siRNA complexes were used as a control in all luciferase silencing studies. Treatment with formulations prepared with negative control (siScr) showed no effect on luciferase expression, confirming safety of the studied systems. Overall, the conjugate polyplexes rPAMD-SS-siRNA exhibited better luciferase silencing activity than rPAMD/siRNA polyplexes at both w/w ratios. As expected polyplexes prepared at w/w 5 showed better silencing activity than those at w/w 2. HSA coating had a small, but significant, positive effect on the silencing activity of the conjugate polyplexes but no effect on the activity of the conventional polyplexes. We found that HSA[rPAMD-SS-siRNA] prepared at w/w 5 showed the best luciferase silencing as they decreased the
expression to ~45%. The best performing conjugate polyplexes showed activity fully comparable to the Oligofectamine control.
Figure 19. Cytotoxicity of polyplexes and conjugate polyplexes in B16F10-luc. Cells were treated with the formulations prepared at (a) w/w 2 and (b) w/w 5. Data are shown as mean viability ± SD (n = 3).
Figure 20. Transfection activity of polyplexes and conjugate polyplexes. Formulations were prepared either with siLuc or control siScr at (a) w/w ratio 2 or (b) w/w ratio 5. Luciferase silencing was determined by in B16F10-luc cells. Data are shown as mean % luciferase expression ± SD (n = 3). * p < 0.05, ** p < 0.01, *** p < 0.001.
3.5 CXCR4 Antagonism and Inhibition of Cancer Cell Invasion

We have developed rPAMD as antimetastatic inhibitors of the chemokine receptor CXCR4 and it was important to validate that the conjugate polyplexes retained the original CXCR4 inhibitory activity. First, we measured CXCR4 inhibition using CXCR4 redistribution assay by a high-content fluorescence microscopy analysis as described previously [259, 260]. We have used a small molecule CXCR4 inhibitor AMD3100 as a positive control and expressed the results as % of the AMD3100 activity. As shown in Figure 21a, we have found that all tested formulations used at concentrations used in the siRNA silencing studies showed CXCR4 inhibition indistinguishable from the activity of 300 nM AMD3100. We then investigated if the demonstrated CXCR4 inhibition resulted in the ability of the polyplexes and conjugate polyplexes to inhibit invasion of cancer cells. The important role of CXCR4 in the migration and invasion of multiple types of cancer cells and the ability of CXCR4 inhibitors to prevent the migration is well known [261]. We have previously shown that both rPAMD and rPAMD/DNA polyplexes effectively inhibit CXCR4-mediated invasion of multiple cancer cells [254, 262]. As shown in Figure 21b, all the polyplexes and conjugate polyplexes showed effective inhibition of cell invasion at w/w ratio either 2 or 5. Based on the quantification of the average numbers of invaded cells, 71% cancer cells were prevented from invading and migrating through the layer of Matrigel by control AMD3100. The polyplexes and conjugate polyplexes achieved similar activity as they inhibited invasion of 60-70% cancer cells. Both of the above experiments together demonstrated that the conjugate polyplexes retain the CXCR4 activity of rPAMD. While the mechanism of the CXCR4 inhibition by the conjugate polyplexes is not fully
understood, we believe that excess rPAMD used in the formulations is most likely responsible for the observed activity. Additional CXCR4 inhibition is likely contributed as rPAMD is released and degraded in the cells following intracellular delivery and disassembly of the conjugate polyplexes.
Figure 21. Inhibition of CXCR4 and cancer cell invasion. (a) CXCR4 inhibition of polyplexes and conjugate polyplexes in U2OS cells. AMD3100 (300nM) was used as a positive control. Data are shown as mean % CXCR4 antagonism relative to AMD3100 ± SD (n = 3). (b) Inhibition of cancer cell invasion. U2OS cells were treated with polyplexes or conjugate polyplexes (rPAMD 2.0 µg/mL) and allowed to invade through Matrigel upon stimulation with SDF-1 for 16 h. AMD3100 (300 nM) was used as positive control. Data are shown as mean number of invaded cells ± SD (n = 3).
3.6 Transfection Activity in vivo

The ability of the conjugate polyplexes to deliver siRNA systemically after intravenous injection was investigated in mice bearing B16F10-luc tumors. We first conducted a dose-finding and preliminary toxicity evaluation of both conventional polyplexes and conjugate polyplexes both with and without HSA coating. We found that coating with HSA improves safety of both formulations and increases the estimated maximum tolerated dose about 2.5-fold (not shown). We thus focused our attention on the HSA-coated formulations. The B16F10-luc cells were injected subcutaneously and the tumor growth was followed by IVIS bioluminescence imaging. We have started the treatments with HSA[rPAMD-SS-siRNA] and HSA[rPAMD/siRNA] when the tumors reached 50 mm³. Both conjugate polyplexes and conventional polyplexes were prepared with siLuc and siScr. The control siScr formulations were injected to establish 100% luciferase expression levels. Following the treatments and shortly before animal sacrifice, whole-body luminescence images were taken (Figure 22a) and luciferase expression in the tumor regions quantified. As shown in Figure 22b, the HSA-coated conjugate polyplexes showed increased luciferase silencing (~50%) when compared with the HSA-coated conventional polyplexes (~30%). The tumors were resected, mechanically homogenized, lysed and the luciferase silencing was validated in the tumor lysates as above (Figure 22c). The tumor lysate results confirmed the superior silencing activity of the conjugate polyplexes. The observed improved in vivo activity of the conjugate complexes confirmed the in vitro findings and showed the importance of the polyplex stabilization for systemic delivery. The primary benefit of coating with albumin appeared to be decrease in systemic
toxicity most likely due to reduction in the surface charge. Potential effects of the HSA coating on pharmacokinetics and renal clearance remain to be investigated.
Figure 22. In vivo luciferase gene silencing by HSA-coated conjugate polyplexes. Mice with subcutaneous B16F10-luc tumors were given daily intravenous injections for 4 days of the HSA-coated polyplexes or conjugate polyplexes prepared with either siLuc or control siScr. (a) Bioluminescence images of the tumor-bearing mice before sacrifice. (b) Luciferase silencing determined from the region of interest analysis of the tumor-associated signal in live animals. Luciferase expression in animals treated with siScr was set as 100%. (c) Luciferase silencing in homogenized tumor tissues. Data are expressed as mean ± SEM (n = 7). * p < 0.05, ** p < 0.01.
4. Conclusions

In conclusion, we described a simple approach to prepare polyplexes stabilized against dissociation using in situ thiol-disulfide exchange between siRNA-SH and bioreducible branched polycations. Our results demonstrated not only increased overall stability of the conjugate polyplexes but also enhanced gene silencing activity in vitro and in vivo. Further stabilization of the polyplexes by surface coating with albumin provided additional enhancement of gene silencing in vitro and decrease of toxicity in vivo. Using polycations that inhibit CXCR4, we confirmed that even when conjugated to the siRNA, these polycations retain their inhibitory activity as suggested by strong ability to prevent invasion of cancer cells. Overall, these initial findings provide impetus for further evaluation of the conjugate polyplexes in systemic anticancer and antimetastatic siRNA therapies. In the future, these innovative conjugate polyplexes can also be evaluated in systemic renoprotection of AKI.

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Chapter 5. Summary and Future Directions

AKI has high mortality and morbidity and remains the significant medical problem in the world. It affects over 13 million patients worldwide every year. People who have AKI may finally have chronic kidney disease or even ultimately kidney failure. However, there are few effective pharmaceutical treatments to prevent AKI due to its complex pathogenesis. It may be necessary to develop dual- or multi-functional methods to simultaneously treat AKI. In recent years, drug targeting to the proximal tubule cells, which are the primary injured site in AKI could provide better treatment by means of enhancing the therapeutic efficacy of drugs and lowering unwanted side effects in other organs. Thus, we first developed a variety of polymers with different physicochemical properties for better understanding how those properties influence the distribution in kidney with AKI. After we successfully synthesized pAPMA, pMAA and pHPMA with different sizes, we found that the negatively charged pMAA-5 and neutral polymer of pHPMA-36 showed increased accumulation in injured kidneys at 24 hours after administration. These results confirmed that different properties of polymers have an effect on their deposition in kidneys.

Cytokines and chemokines are elevated in response to AKI, leading to a proinflammatory microenvironment. Emerging evidence shows that the CXCR4/SDF-1 axis is implicated in regulating trafficking and invasion of inflammatory cells in the injured kidneys and inhibition of the axis exerts beneficial therapeutic effect in experimental AKI animal models. siRNA has been used to successfully ameliorate AKI by inhibiting specific gene expression through hydrodynamic injection. However, the
hydrodynamic injection produces high pressure during administration which will lead to liver damage and local renal dysfunction. Therefore, we developed a novel combination therapeutic strategy for systemic treatment of AKI based on previously synthesized dual-function polymeric CXCR4 inhibitors (PCX) capable of systemically delivering siRNA targeting GAPDH (siGAPDH) to the kidneys with AKI. By using this polymer, we safely and efficiently delivered siRNAs to kidneys. First, we confirmed the increase in CXCR4 level of kidney after I/R surgery. Then we demonstrated that our polymer and polyplexes have the potential to accumulate in kidneys with cisplatin-induced AKI. For therapeutic effect, our anti-CXCR4 polymer demonstrated renoprotective effect on cisplatin induced AKI animals by means of inhibition of CXCR4. In addition, the polyplexes showed increased siRNA silencing efficiency when compared with hydrodynamic injection. By doing so, we successfully ameliorate AKI not only by siRNA silencing, but also through interference of the CXCR4/SDF-1 signaling pathway. This method will be a promising therapeutic way for the treatment of AKI.

Conventional siRNA polyplexes are formed by electrostatic interactions between polycations and the nucleic acid. The cationic carriers may not form sufficiently strong interactions with anionic siRNA simply through electrostatic interactions because of the relatively small size of siRNA. Therefore, one of the major challenges of these polyplexes when applied in vivo is their stability against disassembly with competing polyelectrolytes and other charged molecules in serum. To overcome the limitations of siRNA in effective electrostatic condensation with polycations, we tested a simple thiol-disulfide exchange conjugation strategy to prepare rPAMD-SS-siRNA polyplex conjugates and their stability
in serum and against heparin exchange significantly increased. Reporter gene silencing activity of the polyplex conjugates in B16F10-luc mouse melanoma cells showed a comparable efficiency with commercialized Oligofectamine. Finally, the systemic siRNA delivery efficacy of the developed polyplex conjugates in vivo in a syngeneic tumor model showed a promising silencing effect.

Our future studies will focus on drug delivery using the selected polymers that specifically target kidneys with AKI. On the other hand, we will also improve the polyplex formulations and on the renoprotective effect of these polyplexes in AKI. The pathophysiological roles of the novel pharmacological dual-functional polyplexes will be evaluated in several proteins overexpressed during AKI. Last but not the least, we will improve survival rate of mice with AKI.
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