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Targeted Renal Therapies through Microbubbles and Ultrasound

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Abstract

Microbubbles and ultrasound enhance the cellular uptake of drugs (including gene constructs) into the kidney. Microbubble induced modifications to the size selectivity of the filtration capacity of the kidney may enable drugs to enter previously inaccessible compartments of the kidney. So far, negative renal side-effects such as capillary bleeding have been reported only in rats, with no apparent damage in larger models such as pigs and rabbits.

Although local delivery is accomplished by applying ultrasound only to the target area, efficient delivery using conventional microbubbles has depended on the combined injection of both drugs and microbubbles directly into the renal artery. Conjugation of antibodies to the shell of microbubbles allows for the specific accumulation of microbubbles in the target tissue after intravenous injection. This exciting approach opens new possibilities for both drug delivery and diagnostic ultrasound imaging in the kidney.

Keywords

Microbubbles; Ultrasound; Kidney; Drug delivery; Bioeffects; Contrast agents; Molecular imaging; Targeted microbubbles

1. Introduction

1.1. Microbubble structure and composition

Microbubbles were originally developed as ultrasound contrast agents and are administered intravenously to the systemic circulation to enhance the scattering of blood in echocardiography [1]. Microbubbles consist of a gas core stabilized by a thin shell, and range from 1 to 10 μm in diameter (Fig. 1). Several types of commercial microbubbles are available, and they differ mainly in the composition of the shell and gas. Microbubbles are generally composed of air or high molecular weight gasses such as perfluorocarbons or sulfur hexafluoride. The encapsulating shell can be prepared from denatured protein, biocompatible polymers, phospholipids, or a combination thereof.

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1.2. Acoustic properties of microbubbles

The small size of the microbubbles allows them to oscillate in the ultrasound field of medical ultrasound scanners operating in the 0.2-15 mHz range, providing strong contrast of the perfused blood vessels. Furthermore, resonance of the vibrating microbubbles produces echo signals at frequencies distinct from the frequency of the applied ultrasound. These so called harmonic signals can be used to preferentially enhance the signal of microbubbles over the background signal. At higher ultrasound intensities, microbubbles begin to oscillate violently, resulting in the destruction of the microbubbles, a process known as inertial cavitation. This ability to investigate reperfusion of microbubbles after selective destruction has become an important tool for imaging the reperfusion of tissues, particularly in assessing infarct size in echocardiography [2,3].

1.3 Microbubbles and ultrasound enhance cellular uptake

In addition to their applications in medical and preclinical imaging, numerous studies have demonstrated that microbubbles in combination with ultrasound can greatly enhance the cellular uptake of extracellular molecules. There has however been considerable debate on the exact mechanisms underlying the microbubble and ultrasound mediated cellular uptake of drugs. One popular hypothesis is that destruction of microbubbles in the vicinity of the cell results in the formation of transient pores in the plasmamembrane, allowing temporary passive diffusion of compounds over the plasmamembrane [4]. A recent study from our team demonstrated that this mechanism is only involved in the cellular uptake of small molecules (<70 kDa) while the cellular uptake of larger molecules (70-500 kDa) is mediated exclusively through endocytosis [5].

1.4 Principle of microbubbles and ultrasound mediated local drug release

In addition to the ability of microbubbles and ultrasound to enhance the uptake of drugs to compartments within the cell, microbubbles may also be used to release drugs in the lumen of blood vessel of specific organs or tissues. In this approach, drugs are attached to or contained within the microbubbles. Upon injection, the drugs can be released in the target organ by applying high intensity ultrasound locally (Fig. 2).

To date, ultrasound and microbubble mediated drug therapy has focused on the vascular delivery of plasmids encoding either reporter genes or potent paracrine factors and has been successfully applied in several experimental disease models to promote angiogenesis [6-9], attenuate vascular sclerosis [10], reduce neointima formation [11-13] and augment endothelial function [14]. However, the effects of microbubbles and ultrasound are not only confined to the vascular wall as microbubbles and ultrasound can promote local extravasation sites in the capillaries of the insonated organs or tissues [15,16], allowing the delivery of drugs into the organ tissue.

The benefits of targeted drug delivery using microbubbles and ultrasound are obvious, particularly in kidney disease. Examples include kidney transplantation and kidney fibrosis. The kidney is the most transplanted organ in the US and targeted delivery of immunosuppressive drugs or gene-constructs could greatly reduce the severe side-effect of systemic immunosuppression. In addition progressive chronic kidney diseases are associated with the development of fibrosis processes and there is intense interest to target and inhibit the profibrotic mediators. Among all the potential mediators of fibrosis, it is clear that TGF- β stands out, as highlighted in numerous reviews[17,18]. Neutralization of TGF- β using specific antibody or a soluble TGF- β co-receptor in mice with renal injury could reduce the tissue fibrosis [19-22]. However, clinical trials of direct TGF- β inhibitors have not as yet translated these results to the patient. Moreover these approaches require a large amount of antibodies and unwanted side-effects might be observed as TGF- β is also involved in

numerous physiologic roles. Consequently, the local delivery of TGF- β antagonists through directed ultrasound could reduce the amount of therapeutic antibodies needed as well as reduce severe systemic side-effects. Despite its potential, microbubble and ultrasound mediated local drug delivery is still in its infancy. However, several important steps have been taken over the last years. This review therefore aims at providing an overview of the field of targeted renal therapies through microbubbles and ultrasound.

2. Biodistribution of microbubbles after systemic injection

After injection into the systemic circulation, microbubbles are rapidly cleared from the blood with a typical half-life less than 15 minutes [23]. Loss of gas from the core of the microbubble and non-specific accumulation of microbubbles in liver, lung and spleen are the main mechanisms for the rapid decline in the number of detectable microbubbles circulating in the blood [24-26]. The non-specific accumulation of microbubbles in these organs is mediated through phagocytosis of microbubbles by macrophages and by the lodging of microbubbles in the small or contracted capillaries of these organs. Surprisingly, the relative distribution over the three organs differs between animals and is presumably related to relative differences in the abundance of macrophages in these organs of specific species [27]. Although a thorough review on the biodistribution of microbubbles is beyond the scope of this article, the non-specific accumulation of microbubbles in liver, lung and spleen should be taken into account when designing new microbubble and ultrasound mediated delivery strategies to the kidney.

3. Microbubble behavior in the kidney

In order to use microbubbles for targeting to the kidney, the behavior of microbubbles in the kidney needs to be established.

3.1. Microbubble behavior in the healthy kidney

In the healthy kidney, microbubbles have been frequently used in contrast enhanced ultrasonography, a technique that has improved the ultrasound imaging capabilities of kidneys, particularly in the field of imaging of renal arteries and veins. Furthermore, microbubbles has improved the evaluation of renal tissue perfusion and may be used as an alternative to the conventional laser-doppler methods [28-31]. After intravascular injection into healthy animals or humans, conventional microbubbles do not stick to the wall of renal blood vessels or capillaries and do not enter the renal interstitium. In addition, microbubbles are not filtered and in contrast to the liver, microbubbles are not phagocytosed or otherwise retained in the healthy kidney. Furthermore, as the kidneys receive approximately 25% of the cardiac output, a considerable amount of microbubbles will enter the kidneys after intravenous or arterial injection. Due to these properties, microbubbles do not appear to accumulate in the healthy kidney but may be suitable vectors for targeted drug delivery to the diseased kidney..

3.2. Microbubble behavior in the inflamed kidney

Although microbubbles are not phagocytosed directly by renal cells, activated leukocytes have been reported to take up circulating microbubbles in the inflamed kidney of mice after induction of ischemia-reperfusion injury, resulting in increased echogenicity of the inflamed kidney over the control kidney [32]. Similar results were obtained in the myocardium immediately after cardioplegic arrest in dogs, although here microbubbles were directly injected into the myocardium through arterial injection [33]. The mechanisms for microbubble phagocytosis by leukocytes are initiated through the binding of microbubbles to activated leukocytes adherent to the microvascular wall [34]. Although leukocytes are able to bind both albumin and lipid microbubbles [34,35], the underlying mechanisms are

different, with albumin microbubble uptake being dependent on integrin-mediated binding and the uptake of lipid microbubbles being dependent on complement mediated binding [34]. After phagocytosis, the integrity of the microbubbles is preserved. However, the acoustical properties of integrated microbubbles are modified and can be distinguished from those of non-integrated microbubbles [36]. These differences allow for the identification of activated neutrophils at sites of inflammation using ultrasound imaging [36]. Furthermore, a recent study demonstrated that ultrasound mediated excitation of integrated microbubbles may modify the plasmamembrane permeability of neutrophils thus presenting new strategies to modify inflammatory processes [37]. Despite the phagocytosis of conventional microbubbles by leukocytes in inflamed tissues, the accumulation of microbubbles in inflamed kidneys is rather modest and can be dramatically improved by the use of microbubbles targeted to specific markers of inflammation (see section 6.1.).

4. The effects of ultrasound and microbubbles on renal physiology

4.1. Adverse bioeffects: capillary rupture

The bioeffects of ultrasound in combination with microbubbles have been extensively studied and reviewed (for recent reviews see [38-40]). These studies demonstrated that inertial cavitation of microbubbles in close proximity of the capillary wall can cause microscopic bioeffects, including microvascular leakage, capillary rupture, and local induction of cell death and inflammation. Although these phenomena can occur in several organs, the kidney appears to be especially sensitive to the adverse side-effects of ultrasound and microbubbles. This may be related to the relatively high blood pressure within the capillaries of the glomerulus, resulting in substantial bleeding into the Bowman's space with subsequent loss of function of the whole nephron [41,42]. The capillary rupture may be temporary and reversible, but in approximately 50% of the affected nephrons, tubular necrosis led to permanent loss of function [41]. Although these studies were performed in rats, the geometry of the ultrasound setup, the dose of microbubbles and the ultrasound settings were similar to the conditions of clinical contrast enhanced ultrasound imaging of the human kidney. Therefore, the combination of ultrasound and microbubbles could potentially cause similar damage in the human kidney, especially at higher ultrasound intensities (mechanical indices (MI) >0.6), extended imaging duration and increased microbubble concentration.

In contrast to the rat study, a recent study performed in pigs did not demonstrate kidney damage after in situ kidney insonation using Sonovue microbubbles [43]. This study aimed at establishing acute kidney damage after microbubble insonation in an experimental model that resembled the human kidney more closely. In this study, the ultrasound transducer was directly placed on the surface of the porcine kidney and microbubbles were continuously infused through the femoral vein. Despite the high intensity ultrasound applied to the kidney (MI 1.9) and high concentration of microbubbles, no capillary bleeding or other signs of renal damage could be observed. At present, there is no explanation for the discrepancy between the rat and porcine model. Several differences are present in the experimental setups of both studies, including type of microbubble, ultrasound setup and insonation protocol. Nevertheless, the results of the porcine study in combination with the absence of reported negative side-effects in human contrast enhanced imaging studies of the kidney, suggest that microbubble insonation and inertial cavitation may be safe in the human kidney.

4.2. Changes in renal filtration properties

In addition to causing capillary hemorrhage at higher mechanical indices in rats, a recent study performed in rabbits demonstrated that changes occur in the filtration properties of the kidney after exposure to microbubbles and ultrasound [44]. Urine flow, creatinine clearance

and clearance of 3000-Da dextrans and 70.000-Da dextrans were all increased after microbubble and ultrasound treatment. At present, it is unclear how long these effects last and whether this method could be beneficial to patients with impaired kidney function remains to be established [45]. Nevertheless, this study demonstrates that the size selectivity of the kidney can be influenced by the combination of microbubbles and ultrasound, opening the possibility of delivering higher molecular weight drugs to compartments of the kidney that were previously inaccessible. Furthermore, this study demonstrated only minor tubular damage at the highest power setting (1.7 W). However, the results of this study are difficult to compare to previous studies as the authors used focused US with unknown pressures or mechanical indices in the focal plane. In addition, ultrasound with a frequency of only 0.26 MHz was used in contrast to the more commonly used imaging frequencies between 1 and 20 MHz. It is therefore unclear whether the settings used in the rabbit study could result in substantial inertial cavitation of the microbubbles.

Taken together, these studies demonstrate that the safety of inertial cavitation of a high dose of microbubbles in close proximity of the capillary wall is still under debate. Under these conditions, capillary rupture with subsequent nephron loss can occur in rats. In rats, capillary rupture may be avoided if targeted microbubbles are infused slowly with intermittent ultrasound exposure. Such an approach would avoid too much accumulation of targeted microbubbles in the renal capillaries, while still delivering drugs through microbubble cavitation.

5. Ultrasound mediated renal delivery using untargeted microbubbles

Local drug delivery to the kidney may be accomplished by using ultrasound specifically aimed at the kidney. The local insonation of circulating microbubbles in the kidney would result in microbubble destruction with subsequent delivery of the drugs contained within or attached to the shell of the microbubble (Fig. 2). Although the first studies exploring this concept were performed in the late nineties [46-48], there is little data on how much drugs can actually be delivered to the kidney.

5.1 How much drug can be delivered in the kidney?

In a recent study by Tartis *et al*, the renal deposition of radiolabeled microbubble shell components was quantified using microPET after local ultrasound insonation of one kidney in healthy rats [26]. For this, radiolabeled lipids were integrated into the shell of a lipid microbubble and the generated microbubble suspension was injected intravenously. In the following 20 minutes, cycles of low and high power ultrasound insonation were applied to the kidney to generate a radiation force that moved the microbubbles near to the vascular wall with release of the labeled lipid by subsequent microbubble destruction. Although the insonified kidneys contained significantly more label than the control kidney, only an estimated 0.2 µg of labeled lipid could be deposited in the treated kidney. However, the efficiency of lipid-fusion of microbubble shell components to the plasmamembrane of the cells in the vascular wall is unknown. Therefore, the delivery of high affinity drugs may be more efficient.

5.2 Microbubble and ultrasound mediated delivery of plasmids in the kidney

Despite the relatively low efficiency of ultrasound mediated renal delivery using untargeted optison microbubbles, several studies have shown remarkable results using this approach [49-53]. It should however be noted that in these experiments microbubble suspensions were directly infused into the renal artery and were not given intravenously. Furthermore, the delivered plasmids and oligonucleotides were not attached to the shell of the microbubbles. Using this approach, the glomerular uptake of labeled oligonucleotides could be greatly

enhanced. As a proof of concept, this approach was used with to enhance delivery and expression of a Smad7 expressing plasmid to glomerular cells, capillary endothelial cells and interstitial (myo)fibroblasts. Smad7 is an endogenous inhibitor of TGF- β signaling, a strong pro-fibrotic pathway involved in fibrotic kidney disease. The functionality of the delivered Smad7 construct on inhibiting fibrosis has now been demonstrated in several experimental models of fibrotic kidney disease, including the rat urethra obstruction model [50], the rat 5/6 nephrectomy model [52,53] and the murine autoimmune glomerulonephritis model [49]. These studies did not report evidence of capillary damage caused by microbubble cavitation. However, kidneys were examined 7 to 28 days after ultrasound treatment, at which time the capillary bleeding may have been resolved. In contrast to arterial administration, direct injection of microbubbles and plasmids into the renal-parenchyme does not appear to be efficient in the kidney and resulted in only low expression of luciferase in the mouse kidney, despite the functionality of this approach in muscle and skin [54].

In addition to its application in the kidney, optison microbubbles have now been used successfully for gene transfection in several other tissues, including blood vessels [55,56], skeletal muscle [57], heart [58,59], lung [60], liver [61] and tumors [62,63]. Similarly as in the kidney, optison microbubbles and plasmids were either injected directly into these tissues or infused immediately upstream of the target tissue.

Despite the successes of ultrasound and microbubble mediated Smad7 gene delivery to the kidney, this approach for kidney transfection has not been adopted by other groups. Major abdominal surgery is needed to get access to the renal artery, resulting in a systemic inflammatory response that generally interferes with renal physiology. Further, the local infusion of the mixture of microbubbles and plasmid into the renal artery requires considerable surgical skills when performed in small rodents. These major disadvantages of conventional microbubbles may be largely solved by the development of new approaches for increasing microbubble concentration specifically in the target organ.

6. Ultrasound mediated renal delivery using targeted microbubbles

Intravenous injection into the bloodstream is in general the preferred route for microbubble administration. However, once microbubbles are dispersed over the total blood volume, the concentration of microbubbles drops dramatically. Furthermore, microbubbles and drugs quickly separate after intravenous injection if both are not directly coupled. For this reason, most in-vivo microbubble mediated delivery studies relied on microbubble infusion directly upstream of the target organ (see section 5.2.).

Advances in microbubble technology allowed for the coupling of antibodies and other targeting ligands to the shell of the microbubble, enhancing the retention of the microbubbles in specific organs. This targeted microbubble approach allows for the specific accumulation of targeted microbubbles in the target tissue without the need for specialized surgical procedures. The size of the typical microbubble generally renders it confined to the intravascular space. Thus, relevant molecular targets for this technique must be expressed on the vascular lumen or on intravascular cells.

For drug delivery, drugs may be attached to the outside of the microbubble shell, integrated into the shell or may be contained within the microbubble shell. Regardless of its position, the full release of the drug is only accomplished if the drug carrying microbubbles are destroyed by the local application of high intensity ultrasound in the target organ. Furthermore, it is reasonable that non-specifically accumulated microbubbles in the liver, lung or spleen (see section 2), if not exposed to ultrasound, do not release their drug payload. Therefore this approach would specifically deliver drugs to the target tissue,

contributing to the development of a clinical ultrasound microbubble delivery therapy. In addition, the specific retention of targeted-microbubbles to the diseased tissue, would allow for non-invasive monitoring of the progression of disease using low-power ultrasound-imaging.

6.1. Composition of targeted microbubbles

A popular method for the targeting of microbubbles is the coupling of specific antibodies to microbubbles using a biotin/ streptavidin scaffold (fig. 3). These microbubbles are now commercially available (Targeson) and provide an easy-to-use conjugation system for proof-of-concept work. However, both antibodies and the biotin/ streptavidin scaffold can trigger an immune response[64], making these targeted microbubbles unsuitable for clinical studies. These problems have recently been overcome by the introduction of a biocompatible coupling system based on covalent coupling of targeting entities to the shell of the microbubble [65-68]. Furthermore, targeting of these microbubbles was achieved by humanized binding proteins or receptor specific peptides as an alternative to the immunogenic antibodies.

In a limited number of tissues, targeting of microbubbles can be achieved by simply modifying the chemical properties of the microbubble shell. For example, Sonazoid, a microbubble with a charged shell, is taken up specifically by liver Kupffer cells through phagocytosis[69,70], making these microbubbles suitable for clinical liver imaging[71,72]. Exposure of these phagocytosed microbubbles to ultrasound may even result in therapeutic effects in liver cancer through induction of apoptosis and alteration of gene expression[73].

6.2. Targeted microbubbles in kidney disease

The first attempts to construct microbubbles that could be targeted to specific renal targets were made by Lindner *et al.* in 2000 [32]. Addition of a phosphatidylserine (PS) group to the shell of the microbubble enhanced the complement mediated binding of microbubbles to leukocytes as described in section 3.2.. Evaluation of these PS-microbubbles in a mouse model of kidney inflammation demonstrated a two-fold increase in the number of retained PS-microbubbles in the kidney compared to conventional microbubbles. Furthermore, a good relation was present between the ultrasound signal from the retained PS-microbubbles and the degree of renal inflammation.

The targeting of microbubbles to the inflamed kidney was further enhanced by the ability to couple P-selectin antibodies to the shell of the microbubble [74]. Selectins are anchoring molecules involved in the adhesion and rolling of leukocytes on the endothelium of inflamed tissues. Infusion of the P-selectin microbubbles after renal ischemia-reperfusion injury in mice resulted in enhanced microvascular retention and strong signal enhancement on ultrasound imaging of the inflamed kidney. Although this study demonstrated that renal ischemia-reperfusion injury resulted in the rapid expression of P-selectin on the endothelium of glomerular and peritubular vessels, the exact location of P-selectin microbubble binding was not evaluated.

A recent study aimed at establishing the intrarenal location of P-selectin microbubble binding after renal ischemia-reperfusion in mice [75]. In this study, ischemia-reperfusion injury in the left kidney resulted in increased P-selectin microbubble binding primarily in the corticomedullary junction and to a lesser extent in the cortex. Surprisingly, ischemia of the left kidney resulted in an even more pronounced increase of P-selectin microbubble binding in the contralateral control kidney. These data suggest that P-selectin expression is increased in both kidneys after unilateral induction of renal ischemia. However, the results of this study may be strongly influenced by the severely inhibited renal blood flow to the injured

kidney after induction of ischemia-reperfusion damage, resulting in a decreased level of microbubble entry into the ischemic kidney.

6.3. The effect of blood flow on the binding of targeted microbubbles

Binding of targeted microbubbles to targets within the kidney may be further influenced by differences in local blood flow. In vitro flow chamber experiments demonstrated that increased flow and shear stress can strongly reduce the binding of targeted microbubbles to their targets [76,77]. In conditions of high flow, the application of low-power ultrasound may facilitate the binding of targeted microbubbles by providing an acoustic radiation force that moves the microbubbles out of the center of the bloodstream towards their targets on the vascular endothelium [78]. Despite these efforts of improving microbubble binding in conditions of high blood flow, the effect of (local) blood-flow on targeted microbubble binding should be taken into consideration, especially in the setting of diagnostic ultrasound imaging using targeted microbubbles.

6.4. Local delivery using targeted microbubbles

Although targeted microbubbles have been used primarily in a diagnostic setting in experimental models of inflammation, preliminary data presented at the Fourteenth European Symposium on Ultrasound Contrast Imaging, demonstrates that local plasmid delivery can be achieved with targeted microbubbles [79]. In this study, plasmid bearing microbubbles targeted with anti-mucosal addressin cellular adhesion molecule-1 (MadCAM-1) antibodies accumulated specifically in the inflamed gut of mice with experimental inflammatory bowel disease. Further, subsequent microbubble destruction led to increased plasmid expression in the mouse gut. Importantly, no surgical procedures were needed for the local delivery of plasmids and microbubbles as transfection of the gut was achieved by simple intravenous injection of the plasmid-bearing targeted microbubbles. This technique may greatly improve the use of microbubble mediated delivery, although this concept has not yet been evaluated for targeted delivery to the kidney.

7. Potential renal targets for targeted microbubbles

Inflammation is generally observed in diseased tissues and organs. The inflammatory response is mediated by several receptors found on luminal endothelium [80], which can be readily targeted by ultrasound contrast agents. Active targeting strategies for inflammation have focused primarily upon pro-inflammatory endothelial adhesion molecules. Microbubbles targeted to vascular cell adhesion molecule-1 (VCAM-1) have been used to image atherosclerotic plaque development [81,82]. A similar surface protein, intravascular cell adhesion molecule-1 (ICAM-1; [83]), has been utilized as a molecular target for imaging transplant rejection [84-86] and autoimmune encephalitis [87,88]. Finally, microbubbles targeted to the gut-specific marker MAdCAM-1 were found to be useful for imaging regions of inflammation in a mouse model of Crohn's disease [89]. Until now, targeted microbubbles in renal disease have been primarily aimed at P-selectin in experimental models of renal inflammation after ischemia-reperfusion injury. Furthermore, renal upregulation of P-selectin expression has been described for several other kidney diseases including renal allograft dysfunction [90-92], glomerulonephritis [93-95], renal shock [96,97] and diabetic nephropathy [98,99], indicating that microbubbles targeted to P-selectin may be applied in these kidney diseases. However, the selective targeting of microbubbles to leukocyte anchoring molecules in kidney disease may be compromised when the kidney disease is accompanied by a state of systemic inflammation such as sepsis, reperfusion injury, atherosclerosis and metabolic dysfunction. Furthermore, upregulation of selectins is primarily evident immediately after induction of renal damage, making these

target molecules less suitable for microbubble targeting in chronic models of kidney disease, such as diabetic nephropathy.

A potential new target for microbubble targeting to the kidney was recently identified by our group in an experiment designed at delivering neutralizing anti-TGF- β antibodies specifically to the kidney. The anti-TGF- β antibody used in this study (the pan-specific 2g7 antibody) has previously been shown to be effective in neutralizing the effects of TGF- β in experimental diabetes in mice [20,100]. Interestingly, Targestar microbubbles equipped with the 2g7 anti-TGF- β antibody demonstrated selective accumulation of microbubbles in the diabetic kidney (see Fig. 4, unpublished data). At present, the target cells for these microbubbles are still unknown. Previously, we demonstrated that the chronic type 1 diabetic mouse [100] and the db/db mouse [20] overexpress TGF- β in the glomeruli, most likely through increased production and secretion of latent TGF- β by mesangial cells. Subsequent digestion of this latent complex by various proteases is an essential step to activate TGF- β in diabetic kidney disease. As the 2g7 antibody is specific for active TGF- β [101], our data suggests that active TGF- β is presented on the luminal side of the glomerulus in diabetic mice, where it can act as target for targeted microbubbles. In the same study, microbubbles targeted to P-selectin demonstrated weaker accumulation in the diabetic kidney than TGF- β targeted microbubbles, indicating that TGF- β may be a more suitable target for microbubble targeting in diabetic nephropathy and possibly in fibrotic kidney disease in general. As only a fraction of the antibodies on the microbubble participates in microbubble binding to its target, full release of all the 2g7 antibody would require microbubble destruction by a high power ultrasound pulse. The local release of neutralizing antibodies within the diabetic kidney would reduce the total amount of antibody needed and reduce negative side effects, facilitating new strategies for local neutralization of TGF- β .

In addition to TGF- β , markers associated with neovascularization may be potential targets for directing microbubbles to the kidney. Neovascularization often contributes to renal disease progression or resolution. Microbubbles have been targeted to several molecular markers of angiogenesis, including VEGF/VEGFR2 [102-105] and α -v integrins [106-109]. So far, these targets have not been used for microbubble targeted delivery to the kidney, despite the involvement of these targets in common kidney diseases including renal cancer and diabetic nephropathy [110,111].

8. Summary and future perspectives

Microbubbles and ultrasound show huge promise in enhancing cellular uptake and local drug delivery in the kidney. Microbubbles and ultrasound can be used to modify the size selectivity of the filtration capacity of the kidney, enabling drugs with high molecular weight to enter previously inaccessible compartments of the kidney, including the urinary space containing podocytes and the tubular lumen. So far, negative renal side-effects such as capillary bleeding have been reported only in rats, with no apparent damage in larger models such as pigs and rabbits. This potential to create local extravasation sites may also be employed therapeutically as these sites allow the entry of high molecular weight drugs, antibodies or therapeutic viral constructs into the renal interstitium.

In addition to promoting cellular uptake, microbubbles and ultrasound may be used for local delivery in the kidney. Although local delivery can be accomplished by applying ultrasound only to the target area, efficient delivery using conventional microbubbles has depended on the combined injection of both drugs and microbubbles directly into the renal artery, an invasive technique that requires considerable surgical skills.

Intravenous injection of targeted microbubbles appears to be the most promising technique for local drug delivery in the kidney. Conjugation of targeting ligands and drugs to the shell of microbubbles allows for the specific accumulation of microbubbles in the kidney after intravenous injection. Subsequent exposure to high intensity ultrasound releases the drug from the microbubble. This exciting concept has now been proven efficient in delivering plasmids to the inflamed gut.

The potential for targeted microbubbles is enormous, as microbubbles can now be targeted to specific markers of kidney disease. Until now, only vascular markers of inflammation have been used for renal targeting of microbubbles, but preliminary data suggest that several other factors may be used for microbubble targeting, including growth factors and receptors. The targeted microbubbles can be easily visualized using conventional ultrasound imaging equipment, allowing for both diagnostic imaging and local drug delivery at the same time, something that can not be accomplished by other targeted delivery systems.

Until recently, researchers had to construct their own targeted microbubbles for use in their studies. This technical barrier for obtaining targeted microbubbles has now been overcome as microbubbles with biotin scaffolds are now commercially available (Targeson). Furthermore, ready-to-use targeted microbubbles are now entering the market, such as Bracco's BR55 microbubble directed against VEGF-R2. The emergence of these products will undoubtedly facilitate the use of targeted microbubbles in future targeted delivery studies.

An exciting new field in microbubble research has recently emerged, using the ability of creating microbubbles in-situ from superheated nano-emulsion droplets by the application of ultrasound[112,113]. As these submicron droplets are much smaller than microbubbles, these droplets may be used for drug delivery and ultrasound imaging outside the vascular compartment.

In conclusion, microbubbles and ultrasound have great potential in diagnostic imaging and local drug delivery. Furthermore, the accessibility of renal compartments (including the intracellular compartment) to high molecular weight drugs can be modified by microbubbles and ultrasound.

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Abbreviations

ICAM-1	intravascular cell adhesion molecule-1
MadCam-1	mucosal addressin cellular adhesion molecule-1
MI	mechanical index
PEG	polyethylene glycol
PET	positron emission tomography
PS	phosphatidylserine
TGF-β	transforming growth factor beta
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR2	vascular endothelial growth factor receptor 2

Fig. 1. Structure and appearance of microbubbles. A) Schematic of a phospholipid microbubble. B) Photograph of Sonovue microbubbles (Bracco, Milan) in close proximity to cultured bovine endothelial cells (phase contrast, 400^x magnification). Note that the microbubbles are not uniform in size.

Fig. 2. Schematic diagram demonstrating local delivery of drugs to the kidney using microbubbles and ultrasound. Drug bearing microbubbles (⊛) are injected intravenously into the circulation. Subsequent local exposure of the microbubbles to high intensity ultrasound releases the drug (☆) in the kidney.

Fig. 3.

Diagram of a targeted microbubble. The gas phase is encapsulated by a lipid shell, which is stabilized by a polymer layer. Targeting ligands are immobilized on the distal surface of the polymer using various conjugation strategies, including biotin/avidin coupling, thioether, amide, and disulfide bonding.

Fig. 4.

Representative images of accumulated microbubbles in diabetic mice 10 minutes after microbubble injection. Organ positions were outlined from B-mode images: liver is outlined in green and kidney is outlined in red. Specific accumulation of TGF-beta and P-selectin targeted microbubbles was observed in the diabetic kidney.