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LIVER TRANSPLANTATION (DC MULLIGAN, SECTION EDITOR)

Utilization of Machine Perfusion and Nanotechnology for Liver Transplantation

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Abstract Over the past four decades, advances in the technology supporting solid organ transplantation have been remarkable. Transplantation is now entering a new era where multidisciplinary approaches, including the use of bioengineering, are being utilized in the pursuit of perfection for the field. In this review article, we will introduce and recap two broad categories that are on the verge of revolutionizing the utilization of donor organs and delivery of immunosuppression, metabolic additives, and gene therapies. Machine perfusion techniques and nanotechnology are areas of significant interest in the transplantation community and make up the next generation of bench to bedside research endeavors. In this review, we will summarize the progress made in the field of machine perfusion and nanotechnology as it applies to liver transplantation.

Keywords Machine perfusion · Nanotherapy · Immunosuppression · Transplantation · Donor organ · Liver

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Introduction

As the number of patients awaiting liver transplantation continues to increase, there is pressure on transplant centers to expand the donor pool by using marginal donor organs. Despite this, wait list mortality remains 5–10 % without any significant decrease in the waiting period [1]. Part of the issue relates to the fact that marginal organs, including elderly, steatotic, and organs donated after cardiac death (DCD), have a more limited ability to withstand the damaging effects of cold ischemic time [1, 2•, 3]. Efforts to ameliorate the negative effects of cold ischemia on donor livers, particularly marginal livers, include novel approaches such as ex vivo machine perfusion of the donor organ, and delivery of therapeutics to the donor organ prior to transplantation. These therapeutic strategies lend themselves to nanoparticle-based drug delivery systems, and will be discussed herein.

While these different approaches vary widely in their specifics, the basic premise is the minimization of insults incurred within the donor organ, during storage, and post-transplantation. An additional advantage of these techniques is the ability to monitor donor liver function ex vivo. By appropriating

technology that has been in use with kidney transplantation for many years, machine perfusion of the donated liver aims to stabilize the allograft's microenvironment by removing toxins and providing metabolic supplementation and further provides a window of opportunity for delivery of therapeutics which may impact post-transplant survival [4, 5]. In the early part of this century, animal models were used to demonstrate the ability of hypothermic machine perfusion to ameliorate the effects of ischemia-reperfusion injury, particularly those seen in DCD organs [6, 7, 8•, 9]. In 2010, Guarrera et al. published their experiences with the first human cohort of machine perfused liver transplants, building on previous work they had done in porcine models [5, 9]. Since that time, there has been an exponential growth in research aimed at optimizing the parameters for machine perfusion, the perfusion solutions, and the seemingly endless possibilities for solution additives.

Improvements in donor management techniques have also utilized cutting edge bioengineering such as the use of nanodevices and nanotherapy as a means to deliver agents to improve the longevity and preservation ability of organs. Nanotherapy has recently been utilized by our group and others as a means to deliver therapeutics in the organ preservation phase of transplantation [10••, 11]. Post-transplantation, micro- and nanoparticle based therapies have been employed as the natural evolution of immunosuppressive agents that began with total body irradiation and evolved to calcineurin inhibition and various biologic approaches and now include targeted therapies directed to the organ themselves by focused nanoparticle therapeutics [11]. By approaching the same problem from a different angle, the concept of nanotherapy was aimed at, not only maximizing organ preservation ex vivo, but also at facilitating organ recovery in vivo, with the potential of improving organ quality long-term. Here, we provide an overview of the recent developments in organ protection, with special focus on advancements in machine perfusion and nanotechnology in liver transplantation.

Basics of Machine Perfusion

Machine perfusion (MP) consists of a mechanical pump in circuit with the liver via the hepatic artery and/or the portal vein through which various perfusion solutions can be instilled (Fig. 1) [5, 12]. In comparison to static cold preservation techniques, the continuous flow of MP allows for the washout and removal of accumulated metabolites, as well as enhanced delivery of oxygen and nutrients while ex vivo [5, 12, 13]. The importance of simultaneously perfusing via the hepatic artery and the portal vein should not be underestimated, as both systems of circulation contribute significantly to liver blood flow in vivo. In the setting of ex vivo ischemia, particularly for marginal organs, the absence of either may increase the incidence of post-transplantation complications including delayed graft function, non-anastomotic/intrahepatic biliary strictures, and cholangiopathy [5, 14–16].

Generally speaking, pressure control perfusion has been more widely used than flow control perfusion, with the prevailing opinion being that pressure control will better limit the degree of endothelial shear injury [7, 16–18]. Although the ideal perfusion pressures remain unknown, studies have conclusively demonstrated the deleterious effects of high pressures [7, 18]. While in vivo hepatic artery pressure mirrors systemic mean arterial pressure (70–100 mmHg) and normal portal venous pressure ranges between 5 and 10 mmHg, most liver perfusion systems operate at pressures approximately 25 % of these values based on work by 't Hart et al. demonstrating that in rat models this ratio led to improved outcomes for hypothermic machine perfusion (HMP) in comparison to static solution and 50 % normal systemic pressures [18]. It is important to note that the current pumps and protocols have been designed and optimized around pulsatile flow mechanics in the hepatic arterial system and continuous non-pulsatile flow in the portal venous system [17, 19].

Timing and Duration

The ideal initiation time and duration for machine perfusion of donated livers is still under active investigation. In the initial human trials, Guarrera et al. initiated perfusion after transport of the organ to their facility and the total time on the circuit ranged from 3 to 7 h with total ischemic time maintained under 12 h [5]. As pumps have become more portable, there appears to be a trend toward initiating MP at the donor hospital in an effort to maximize the documented beneficial effects and minimize the off pump cold ischemic time. However, earlier initiation and longer duration of MP will need to be balanced against cost effectiveness if it is to gain widespread popularity. Real time assessment of organ function while on MP should aid efforts toward determining optimal timing and duration of MP in liver transplantation.

Hypothermic Machine Perfusion

The most extensively studied variable of MP is the temperature at which the organ is best preserved. Logically, the initial attempts at transitioning from static cold storage to MP employed a hypothermic perfusion solution at approximately 4 ° C. It was under hypothermic machine perfusion (HMP) conditions that ideal perfusion pressures were first established in animal models [18, 20]. Studies going back as far as 2000 have demonstrated the incremental benefit of HMP in marginal porcine livers [20–22]. Indeed, several injurious pathways related to ischemia-reperfusion appear to be attenuated by HMP [23]. In human models, studies have shown that HMP performed for up to 7 h prior to transplantation can not only

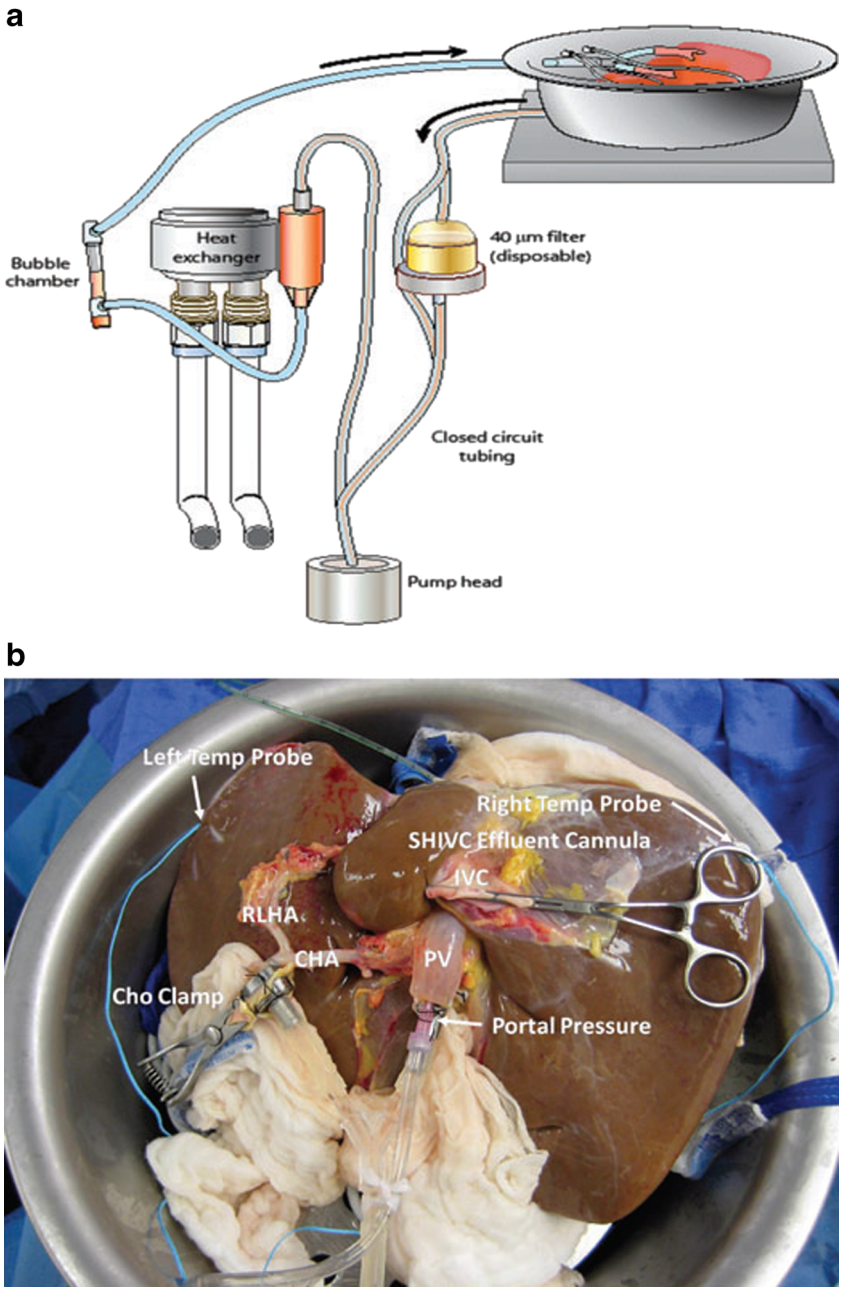

Fig. 1 **a** Schematic diagram of a hypothermic machine perfusion setup. **b** Liver graft during hypothermic machine perfusion. *SHIVC* suprahepatic inferior vena cava, *PV* portal vein, *CHA* common hepatic artery, *RLHA* replaced left hepatic artery. (Reprinted with permission from Am J Transplant, 2010. 10(2): p. 372–81. Copyright 2010 American Journal of Transplantation.) [5]

improve and shorten the post-operative course, but can be used to "recover" marginal livers that had been previously rejected by the United Network for Organ Sharing (UNOS) regional centers. As compared to a control group, there were significant improvements noted in both early allograft dysfunction and biliary complications for HMP livers [5, 24]. The heterogeneity of HMP protocols explains, at least in part, the observation that not all studies of HMP have demonstrated clear benefit. In hypothermic perfusion, there still remains the possibility of functional monitoring of hepatocellular enzymes, and markers of inflammation and cell death, although the implications of these remain less clear. Further correlation to actual transplant models will be required though before these parameters can become standardized.

Additional avenues for the optimization of HMP have included the exploration of oxygenated HMP circuits. Some debate still exists regarding the need for oxygen during hypothermia, with the major concern revolving around the generation of free radicals in an environment already susceptible to ischemia-reperfusion injury [13]. To date, multiple animal studies have documented the potential upside to oxygenation of perfusion solutions for HMP, including prevention of mitochrondrial dysfunction, as well as an overall preservation of adenosine triphosphate (ATP) stores [6, 13, 25–27]. The

concerns over reactive oxygen species production were not realized in these studies, and in fact, Luer et al. achieved optimal results and efficiency in their HMP rat model with 100 % oxygenation of their preservation solution [25].

Normothermic Machine Perfusion

Normothermic machine perfusion (NMP) is an emerging variant of MP that is being actively studied in all solid organ transplantation. The driving hypothesis of NMP is that allowing the organ to function at its in vivo temperature and full (or near full) metabolic capacity will minimize injury, maximize preservation, and optimize healing. A recent series of age-matched expanded criteria donor kidneys preserved under NMP conditions as compared with static cold storage revealed no difference in graft survival at 1 year; however, at 7 days, delayed graft function rates for the NMP perfused kidneys were 5.6 versus 36.2 % in the static cold storage control group [28]. Based, in part, on these data, MP technology at normothermic temperatures is presently being explored for use in liver transplantation. It has been shown in discarded human donor livers that under normothermic machine perfusion (NMP) conditions, bile production may represent an appropriate surrogate for viability [2•, 18]. Bile production constitutes a highly complex metabolic process, and indeed, a lack of intraoperative bile production by graft has been identified as an indicator for poor outcomes [19].

Recently, safety and efficacy of NMP was demonstrated in a porcine model of DCD liver transplantation where the livers were maintained for 10 h under NMP conditions following 1 h of warm ischemia. Following NMP treatment or cold storage, the porcine livers were subjected to a 24-h transplant simulation model. NMP-treated organs revealed improved liver function and preserved histologic architecture when compared to static hypothermic storage which suggested irrecoverable injury [29]. Many of the initial studies examining NMP utilized a perfusate consisting of either whole blood or a dilution of whole blood. Blood was the perfusate of choice due to its high oxygen carrying capacity and the idea that the high metabolic demands of the organ under NMP conditions could not otherwise be met. It has been subsequently shown by Boehnert et al. that acellular ex vivo perfusion can improve markers of ischemic hepatic injury [16, 30, 31]. This may offer an opportunity to simplify current NMP protocols that presently require complex systems. However, the necessity of an oxygenator means that the majority of circuits will remain too bulky for easy transport, limiting the potential utility of NMP [30, 32]. Multiple randomized control trials are currently being conducted that may shed light on the progress being made. Utilization of NMP may be beneficial in marginal steatotic organs, as recent studies from the University of Oxford suggest that normothermic perfusion may maintain Factor V levels and bile production in NMP-treated steatotic livers while decreasing their fat content on Oil-Red-O staining in a pig model [33]. While NMP is required for bile production and the degree of biliary conservation achieved by NMP has been well documented, the complexity involved in maintaining the metabolic capacity of the liver ex vivo for a prolonged period of time continues to be a tremendous hurdle to its wide-spread adoption.

Subnormothermic Machine Perfusion

Between HMP and NMP lies subnormothermic machine perfusion that generally calls for temperatures between 20 and 30°C. Like NMP, subnormothermic machine perfusion (SNMP) allows for a degree of metabolic activity that allows for viability testing and ex vivo monitoring of graft function [34]. The biliary preservation seen with NMP models has also been documented in porcine models of SNMP utilizing DCD livers [35]. The primary advantage of SNMP is that the metabolic demands of the organ are only a fraction of those seen with NMP. These lower metabolic demands allow for simplified maintenance of grafts, obviates the need for oxygen carriers in the perfusate, and allows for a more basic perfusate formula in general. Additionally, perfusion parameters at room temperature have allowed for dramatic simplification of the circuit [34, 35, 36•, 37, 38]. SNMP has shown great promise thus far, and appears to incorporate the benefits of both HMP and NMP, while streamlining the circuit and graft maintenance. As the newest of the three modalities, SNMP requires further study.

The Emerging Potential of Nanotherapy in Liver Transplantation

The use of nanotechnology in transplantation represents the "new era" of therapeutic strategies designed to evade the immune system. Nanotechnology allows for flexibility in delivery, uptake, and maintenance of drug levels over time, by engineered controlled release. The potential for nanotherapies to provide energy, metabolic components, anti-oxidants, and gene therapies to donor organs is limitless; however, to date, there is sparse literature focusing on nanotherapies in the arena of transplantation. Although the concept of nanotherapy-based targeted drug and gene delivery in the oncologic literature has enjoyed much attention, nanotherpaeutics are still considered an emerging concept in transplantation. Much of the data that exists in the transplantation literature has predominantly focused on the delivery of immunosuppressants.

Great strides have been made in the field of material science to allow for the potential of conventional medications to be packaged in newer delivery vehicles. These biodegradable nanoparticles may allow for stealth protection of therapeutic payloads while offering advantages over the current standard of care. Alterations in uptake kinetics, along with targeted

drug delivery are only some examples of the potential of nanotherapy in transplantation. Biologic nanoparticles carrying various immunosuppressive payloads could also be used in donor organ management as a potential to pre-treat organs prior to their implantation, thereby blunting the inevitable effects of ischemia-reperfusion injury and allograft rejection [10••]. Additional advantages of nanoparticle-based targeted drug delivery, whether administered to the organ itself or to the patient post implantation, include the ability to maintain therapeutic levels of immunosuppression in the graft or lymph nodes [39].

Distinct opportunities with nanotechnology exist in liver transplantation and include, but are not limited to the following phases: expansion of the donor pool, protection from ischemia-reperfusion injury, and post-transplantation graft protection. Efficient utilization of existing FDA-approved pharmacotherapies by modifying toxicity profiles and delivery strategies via nano-based approaches is on the immediate horizon as the new frontier in transplantation in all three of these phases.

Nanotherapeutic Targeted Drug Delivery

Immunosuppressant medications globally suppress the immune system and have a host of harmful side effects. The systemic consequences of certain drugs are serious enough that they are seldom used in the perioperative period [40, 41]. There is emerging evidence that some of these medications may be beneficial when delivered directly to an allograft thereby accentuating the immunosuppressive effects on the organ and ameliorating the undesirable systemic effects [42]. Additionally, alterations in the chemical composition of nanoparticles can allow for specified intracellular uptake and sustained release kinetics improving their versatility and function. The size of nanoparticles vary from large liposomes (>100 nm) to small micelles (10–15 nm) which also affect their functional ability [43]. Larger liposomes may act as a reservoir for their payloads allowing for sustained release while smaller nanoparticles can alter the function of endothelial cells and dendritic cells by intracellular uptake, for example [44]. Antibodies and amino acid sequences can also be used to decorate these particles for specific targeting purposes with fluorophores used for drug tracking.

Although there are no nano-based therapies that are currently in clinical use for liver transplantation, promising experimental results give hope that liver-targeted nanotherapeutics are imminent. For example, recent studies suggest that hepatocyte growth factor-loaded chitosan nanoparticles improves liver regeneration, liver function, and survival, in rat models of acute liver failure [45]. Further, various immunosuppression regimes utilized in liver transplantation have been studied as a delivery payload by nanodevices. Of these, rapamycin seems to be an ideal test drug as it is sporadically used in the perioperative period due to its systemic consequences. Rapamycin is an approved therapy with a significant side effect profile particularly in the perioperative period. However, rapamycin has been attributed to conferring tolerogenic phenotypes by allowing for the expansion of regulatory T cells in various in vitro and in vivo experimental models as well as inhibiting the maturation of antigen-presenting dendritic cells [46–48]. The encapsulation of rapamycin in micelle nanoparticles with the purpose of delivering the drug to the lymph nodes in different mouse models has recently been published to demonstrate proof-of-concept [49, 50]. In fact, our group has developed a novel delivery method wherein an immunotherapeutic encapsulated in a biologically inert nanoparticle may be delivered to an allograft ex vivo in a perfusion solution prior to implantation (Fig. 2) [10••]. The potential goal of these therapies is to minimize the harmful systemic side effects of traditional pharmacotherapies while allowing for the development of a local microenvironment of tolerance. The ability to modify nanoparticles can facilitate cellular uptake and targeting both ex vivo and in vivo. To this end, our own studies have utilized targeting moieties, such as the amino acid sequence Arg-Gly-Asp (RGD), as a means to facilitate endothelial cell nanoparticle uptake [10••, 51]. Although RGD is nonspecific in vivo, the ex vivo use of RGD-conjugated nanoparticles as an additive to hypothermic storage solutions allows for improved nanoparticle uptake, as compared to their untargeted counterparts [10••]. These data set the stage for application of these novel therapeutic nanoparticles in in vivo studies.

Expansion of the Donor Pool

In addition to targeting specific payloads to the liver allograft for the post-transplant modification of rejection or ischemic injury, certain iterations of these nanoparticles could be potentially utilized to expand the donor pool to marginal organs. Recent literature suggests the ability to perform “ex vivo” repairs on donor organ using gene therapy as well and pharmacotherapeutics. In fact, targeted gene repair using DNA/RNA oligonucleotides delivered via targeted nanocarriers has been suggested in the potential treatment for acute liver failure [52]. The clinical translation of this approach in the lung transplantation has gained much more momentum recently, however. Elegant studies and clinical trials led by Keshavjee and his group at Toronto have proven that the pool of marginal lung allografts may indeed be extended with the use of ex vivo lung perfusion techniques which may include drug and gene delivery for repair [53]. Although not yet a reality in the arena of liver transplantation, the use of nanotherapy as a delivery tool to rehabilitate poor donor allografts may represent the future of donor organ management.

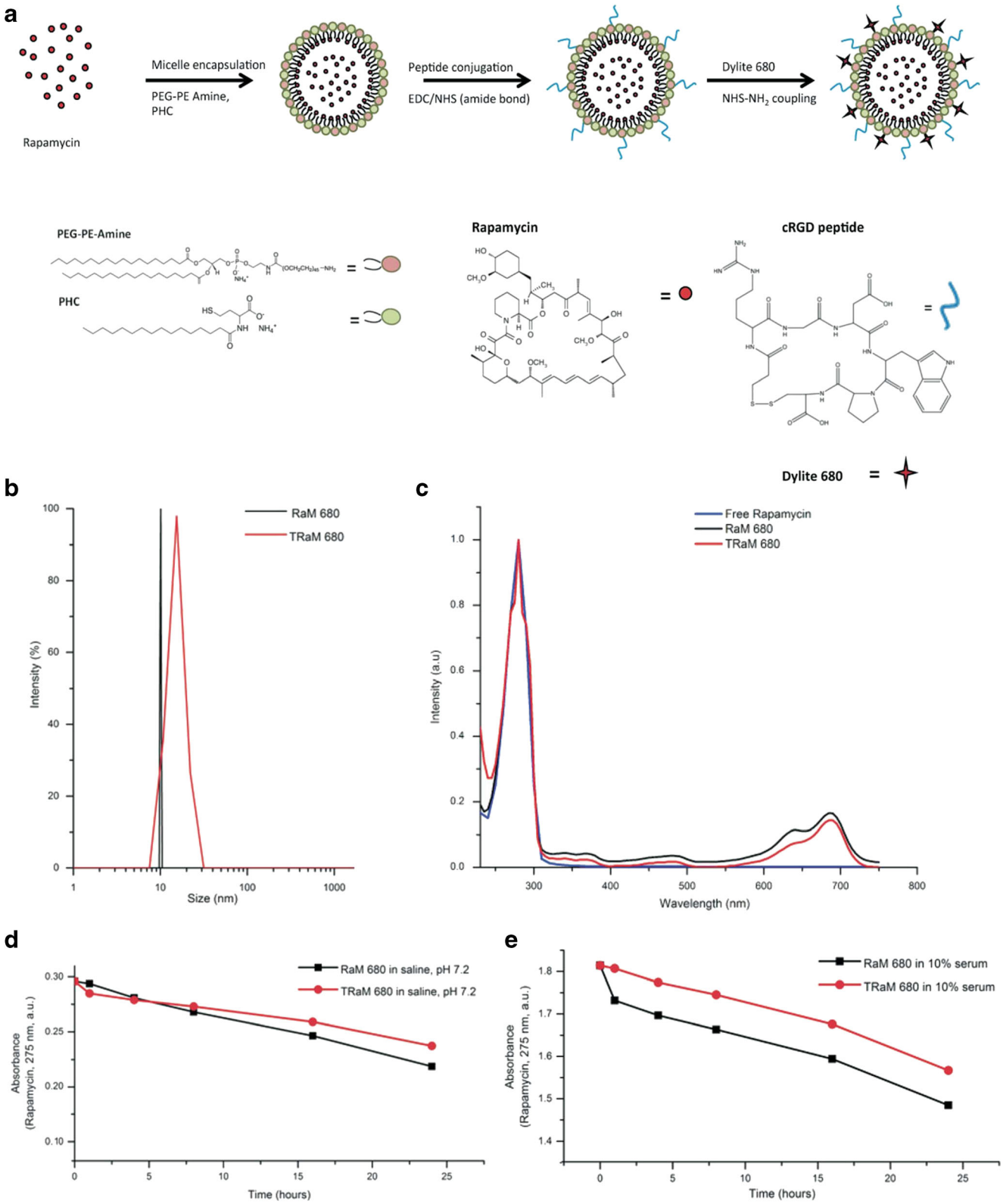

Fig. 2 Fabrication and characterization of rapamycin micelles. **a** TRaMs are composed of rapamycin, NIR fluorophore (Dylight 680), and cRGD peptide targeting moiety for tracking and targeting purposes, respectively. **b** Size calculation using DLS of RaM and TRaM demonstrates micelle sizes between 10 and 12 nm. **c** UV-vis spectroscopy of free rapamycin, RaM, and TRaM identifies rapamycin (275 nm) and Dylight 680 (692 nm). Concentration of each batch calculated based on the rapamycin peak, **d** and **e**, RaM and TRaM were assessed for stability over time in both phosphate-buffered saline and serum, respectively. Both NPs were able to maintain their composition over a 24-h period. (Reprinted with permission from RSC Advances, 2015. 5(54): p. 43552–43562. Copyright 2015 Royal Society of Chemistry.) [10••]

Protection from Ischemia-Reperfusion Injury

Ischemia-reperfusion injury invariably affects all transplanted organs. However, the degree to which the effects cause long-term damage is variable. It is becoming increasingly clear that early injury responses can affect late graft outcomes. Donor organs are exposed to a series of injurious events prior to and during the transplant operative period: brain death, cold storage, cold and warm ischemia-reperfusion, which damage and immunologically prime the donor organ for alloimmune recognition. Central to these injuries is the activation of donor endothelial cells (ECs) and toll-like receptors that, upon reperfusion, promote inflammation, and cytokine release. In addition to these innate immune functions, ECs are central to the recruitment, activation, and even activation of adaptive responses eliciting the proliferation of T and B cells early post-transplantation [54, 55]. With these effects in mind, the innate responses elicited by an allograft upon ischemia-reperfusion injury have been utilized as a target for nano-based therapies. As a proof-of-concept, polymeric nanoparticles intended to protect islet allografts by delivering anti-inflammatory agents revealed remarkable results improving islet endothelial cell binding 3-fold and a 200-fold increase in anti-inflammatory effects with the use of targeted nanotherapy [56]. These results, along with our own, show promise in the development of nanotherapeutics to protect liver allografts in the initial phases post-reperfusion.

Graft Protection Post-Transplant

Many therapies exist as the standard of care to blunt the immunologic responsiveness of liver allografts in the post-transplant period. However, as discussed, the deleterious side effects of these medications often lead to life-threatening and sometimes fatal illnesses. Nanoparticle delivery of drug and gene therapies as immunomodulatory agents or to boost populations of tolerogenic T cells is the next generation of immune maintenance therapy. The spectrum of possibilities is seemingly endless and spans the use of small interfering RNA (siRNA) sequences to silence specific inflammatory gene expression to the use of mToR inhibitors to bolster regulatory T cell populations [11]. Decreasing the antigen presentation capabilities of the host, expanding immunoregulatory cells, or maintaining the immaturity of dendritic cells are only some examples of how nanotherapy in transplantation can lead to the promotion of tolerance [48, 57]. These are among the list of potential targets that bioengineers along with immunologists and transplant physicians are currently focusing on in the interest of developing novel nanocarriers to deliver therapeutics in order to achieve specified immunologic results while obviating systemic side effects.

Along very similar lines, Gajanayake et al. demonstrated the efficacy of a single dose of tacrolimus-loaded hydrogel particles in preventing rejection in rat models of vascularized composite allotransplants (e.g., limb transplants). Hydrogel particles were designed to release tacrolimus in response to upregulated proteolytic enzymes present in the acute post-transplant inflammatory period. The experimental allografts demonstrated not only an extended length of survival (>100 days), but were also shown to have mild-to-absent evidence of rejection. For comparison, rats undergoing a similar limb transplantation with systemic tacrolimus immunosuppression for a 14-day period had a mean graft survival time of 28 days, and received a larger total tacrolimus dose over the treatment period than in the nanogel study (25 vs 7 mg) [58••].

Clinically, the use of a novel targeted immunosuppressant delivery method could potentially alleviate the side effect profile of systemic immunosuppression by allowing for the focused delivery of lower therapeutic drug doses. The use of rapamycin may allow for local tolerance obviating the need for long-term, high-dose immunosuppression. Additionally, the storage and perfusion of organs preservation solutions enhanced with nanotherapeutics prior to transplantation may further minimize toxicity, and potentially provide organs with a level of protection from the inevitable mechanical and immunologic insults that occur during cold ischemia and reperfusion. The translational capacity of nanotherapy in protecting liver allografts post-transplantation is promising both scientifically and economically and may develop in parallel to the discoveries of newer more potent immunomodulatory options.

Conclusions

As transplant waiting lists continue to grow in the setting of fewer available, adequate organs, technologies to improve and expand the current donor pool are becoming more important than ever. Machine perfusion technology has shown great promise in its various iterations at improving the performance of marginal organs, while the utilization of nanotherapeutics may be able to extend the life of these organs and optimize their in vivo performances. By allowing for the use of more efficient doses of organ-specific immunosuppressive medications, while potentially eliminating concomitant side effect profiles, targeted drug delivery offers a more effective and safer environment for marginal organs to survive. As both of these technologies flourish, there will inevitably be greater overlap in the way of nanoparticle containing perfusate, thereby decreasing side effects further. Collectively, these advancements represent realistic solutions for addressing the increasing organ shortage and improving graft outcomes.

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Compliance with Ethics Guidelines

Conflict of Interest Kunal J. Patel, John W. McGillicuddy, and Kenneth D. Chavin declare that they have no conflict of interest.

Carl Atkinson, Ann-Marie Broome, and Satish N. Nadig are co-founders of ToleRaM Nanotech, LLC and hold the executive offices of Chief Scientific Officer, Chief Executive Officer, and Chief Medical Officer, respectively. They also have a patent on Targeted Nanocarriers for the Administration of Immunosuppressive Agents licensed to ToleRaM Nanotech, LLC, and a patent Donor Organ Preservation Immunosuppression with Targeted Rapamycin Micelles (TRaM) licensed to ToleRaM Nanotech, LLC. The company did not fund any of the research presented in this review.

Human and Animal Rights and Informed Consent All original animal work presented in this review performed by the authors is compliant with IACUC guidelines AR#3288. This article does not contain any studies with human subjects performed by any of the authors.

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