Evaluation of Hyperbranched Polyglycerol as a Novel Osmotic Agent for Use in Peritoneal Dialysis

by

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Abstract

Peritoneal Dialysis (PD) is an effective method of renal replacement therapy for patients with end-stage renal disease. PD solution is instilled into the peritoneal cavity and water, solutes, and waste products are removed across the peritoneal membrane, which serves as a natural filter between the peritoneal cavity and the bloodstream. The current conventional PD solution uses hypertonic glucose as an osmotic agent to remove water – a process termed ultrafiltration (UF). Although effective, chronic daily exposure to glucose causes systemic metabolic complications for PD patients; it also directly damages the peritoneal membrane and eventually causes the filter to fail.

Hyperbranched Polyglycerol (HPG) is a non-toxic, non-immunogenic synthetic polymer that contains no starch or glucose. HPG has shown very limited organ accumulation after intravenous injection. HPG offers many theoretical advantages over glucose-based PD including the ability to synthesize HPG over a range of molecular weights. This current thesis tests HPG as a glucose-sparing osmotic agent in PD solution.

We used a rodent model of PD to evaluate solute and waste removal, ultrafiltration, and peritoneal biocompatibility over 0-8 hours of peritoneal exposure. We compared HPG solutions of molecular weights 0.5, 1, and 3 kDa with conventional glucose-based solution (Dianeal™ 2.5%) and buffered glucose-based solution (Physioneal™ 2.27%). We demonstrated that HPG solutions can induce superior and sustained UF for 8 hours, in contrast to glucose-based solutions that lose UF capacity after 4 hours. Sodium and urea removal was superior for HPG solutions, in part because HPG polymer acts as a colloid - as opposed to crystalloid - osmotic agent. We used neutrophil infiltration and peritoneal mesothelial cell detachment as markers of biocompatibility. We found that

HPG solutions, particularly lower molecular weight polymers, demonstrate superior biocompatibility profiles when compared to glucose-based PD solutions.

Taken together, these experiments support the proof-of concept of HPG as a promising novel osmotic agent in PD. Future studies are required to investigate the chronic effects of HPG exposure on the peritoneal membrane, as well as the metabolic and pharmacokinetic profiles of HPG PD solutions.

Preface

This thesis is the product of a collaborative research group consisting of Drs. Gerald Da Roza (clinical nephrologist), Jayachandran Kizhakkedathu (polymer chemist), Caigan Du (experimental biologist/preclinical scientist), and myself (Asher Mendelson - clinician scientist).

Dr. Da Roza and myself conceived the experimental concept for this thesis – polymer-based peritoneal dialysis. After sufficient background research, we approached Dr. Kizhakkedathu to further explore this topic. Together, we identified hyperbranched polyglycerol as a suitable candidate polymer for experimental testing. We then approached Dr. Du and began the preclinical testing of polymer-based peritoneal dialysis in his laboratory.

Under the supervision of Drs Du, Kizhakkedathu, and Da Roza, I have been responsible for the experimental design, data analysis, and data interpretation for all the content described in this thesis.

Under the supervision of Dr. Kizhakkedathu, Ms. Ireena Chafeeva (laboratory technician) was responsible for the synthesis of the polymer used in the experiments.

Under the supervision of Dr. Du, Ms. Qiunong Guan (laboratory technician and animal care specialist) has executed the experiments described in this thesis.

A version of Chapter 2 and 3 is published in *Peritoneal Dialysis International* (Mendelson et al, 2013). I was the primary author on this paper, majority contributor of the content, and ultimately responsible for the final organization of manuscript. Drs Du, Kizhakkedathu, and Da Roza collaborated on data interpretation and manuscript writing and reviewed the final draft. Dr. Du and Ms. Guan executed the experiments described in

the paper. Dr. Kizhakkedathu and Ms. Chafeeva synthesized the polymer used in the experiments.

Most sections of the Chapter 1 consist, in part, of text that has been submitted by our research group for various grant proposals (Kidney Foundation of Canada 2011, 2012; CIHR 2012). I have been a co-investigator on all of the grant submissions and have been the primary author for the text that has been included in this thesis. The technical details of Section 1.9 were supplied by Dr. Kizhakkedathu.

Animal experiments in this thesis were performed in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal Use Subcommittee at the University of British Columbia (protocol #A10-0344).

Publications Arising from Work in this Thesis:

Chapter 2, Chapter 3

Mendelson AA, Guan Q, Chafeeva I, da Roza GA, Kizhakkedathu JN, Du C. Hyperbranched polyglycerol is an efficacious and biocompatible novel osmotic agent in a rodent model of peritoneal dialysis. Perit Dial Int. 2013 Jan;33(1):15-27

In conjunction with the UBC University Industry Liaison Office (UILO), Drs Mendelson, Da Roza, Du, and Kizhakkedathu have filed a US PCT patent application based on the technology described in this thesis.

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List of Symbols and Abbreviations

%FWT = Percent Free Water Transport
σ = Reflection Coefficient
3.86% G = 3.86% Glucose-based PD Solution
Å = Angstrom
AGE = Advanced Glycosylation End-product
ANOVA = Analysis of Variance
AQP1 = Aquaporin 1
CA-125 = Cancer Antigen 125
$CaCl_2$ = Calcium Chloride
CAPD = Continuous Ambulation Peritoneal Dialysis
CKD = Chronic Kidney Disease
D = Dialysate concentration
D/P ration = Dialysate-to-Plasma Ratio
Dn/Dc = Differential Index of Refraction
EMT = Epithelial to Mesenchymal Transition
ESRD = End-stage Renal Disease
FACS = Fluorescence-Activated Cell Sorting
FITC = Fluorescein Isothiocyanate
FSC = Forward Scatter
FWT = Free Water Transport
GDP = Glucose Degredation Products
GFR = Glomerular Filtration Rate
GPC = Gel Permeation Chromatography
H&E = Hematoxylin and Eosin
HBME-1 = Human Bone Marrow Endothelial Cell 1
HD = Hemodialysis
HPG = Hyperbranched Polyglycerol
HPMC = Human Peritoneal Mesothelial Cell
ICO = Icodextrin PD Solution
kDa = Kilodalton
MC = Mesothelial Cell
$MgCl_2$ = Magnesium Chloride
MGG = May-Grünwald-Giemsa
Mn = Number of Average Molecular Weight
mW = Molecular Weight
Mw = Weight of Average Molecular Weight
$NaC_3H_5O_3$ = Sodium Lactate
NaCl = Sodium Chloride
$NaHCO_3$ = Sodium Bicarbonate
NaR = Sodium Removal
Na_t = Sodium Removal at time *t*
PBS = Phosphate Buffered Saline
PD = Peritoneal Dialysis
PDI = Mw/Mn = Polydispersity Index

PDS = Dianeal™ 2.5% PD4 CAPD solution, Baxter Healthcare Co.
PYS = Physioneal™ 2.27% PD4 CAPD solution, Baxter Healthcare Co.
RRT = Renal Replacement Therapy
SD = Standard Deviation
SSC = Side Scatter
TGF-β = Transforming Growth Factor Beta
TPM = Three Pore Model
UF = Ultrafiltration
UFF = Ultrafiltration Failure
UFSP = Ultrafiltration Through Small Pores
V = Volume of recovered Dialysate
VEGF = Vascular Endothelial Growth Factor
$Volume_{dialysate} \times [Na_{dialysate}]$ = Volume of Dialysate multiplied by concentration of sodium in dailysiate

Acknowledgements

I would like to thank the members of my research group for supporting this work, and particularly Dr. Caigan Du for his mentorship, patience, and dedication. I have learned so much about the scientific process and the value of interdisciplinary collaboration. This project has fueled my ongoing passion for research and I am grateful to have shared this experience with such an inspiring team.

This work would not have been possible without support from the BC Provincial Renal Agency. Dr. Paul Taylor and the PD group were instrumental in securing funding for the seed experiments that have subsequently developed into our research program. I would like to sincerely thank Dr. Adeera Levin who has served as my mentor and supervisor in the UBC Department of Medicine. In addition to being a role model, clinician-scientist, and world-renown expert, she has provided me with unwavering support and freedom to explore this research question, and has allowed me the opportunity to connect with an international network of experts in the field.

Dr. Graydon Meneilly, Dr. Anita Palepu, Dr. Mark Roberts, Hazel Wilcox and the UBC Department of Medicine were courageous enough to take a chance so that I could be the first in-training medical resident to pursue a graduate degree. I hope that this work can serve as an inspiration for future residents in the program who wish to pursue careers in academic medicine.

Dedication

I dedicate this work to my parents who taught me to strive to make a difference in this world, to Dr. Bengt Rippe whose work unlocked for me the magic of the peritoneal membrane, and to anybody who believes that just because something has never been done before, does not mean it cannot be done now.

1. Introduction

1.1 Chronic Kidney Disease (CKD) – Overview

Chronic kidney disease (CKD) is defined as the presence of renal damage for a period of greater than three months (1). CKD can be classified in stages 1-5 with each stage representing a decline in the functional ability of the kidneys, as measured by the Glomerular Filtration Rate (GFR) mL/min/1.73m2 (**Table 1.1**). Although CKD can be caused by a variety acute and chronic medical conditions ranging from congenital renal malformations to immune-complex vasculitis, the majority of cases of CKD are attributed to either diabetes or hypertension (2). The prevalence of CKD in the general population of the United States in 2010 is 14.0% (3).

Stage	Description	GFR (mL/min/1.73m2)
1	Kidney damage with normal or increased GFR	≥ 90
2	Kidney damage with mildly decreased GFR	60-89
3	Kidney damage with moderately decreased GFR	30-59
4	Kidney damage with severely decreased GFR	15-29
5	Kidney Failure	< 15 or dialysis

Table 1.1 Stages of chronic kidney disease

The decline in renal function is accompanied by complex metabolic changes for CKD patients. Problems with dyslipidemia and inadequate handling of volume status contribute to increasing cardiovascular mortality in this population (4, 5). Anemia is the result of multifactorial insults to the erythropoietin and iron metabolism systems and is ubiquitous in the severe stages of CKD. Anemia of CKD causes both a reduction in quality of life and all-cause survival for CKD patients (6). Loss of renal filtration ability

leads to severe derangement in serum levels of calcium and phosphate, with subsequent dysregulation of Vitamin D, parathyroid and bone metabolism (7). Moreover, the accumulation of unfiltered nitrogenous "uremic toxins" in the bloodstream is a significant but poorly understood manifestation of CKD (8). These toxins have implications in the overall morbidity and mortality of CKD patients; they affect all systems of the body including leukocyte biology, vascular endothelial tone, neurocognitive function, and glucose homeostasis. As CKD progresses and GFR deteriorates, patients experience an increased risk of death or hospitalization from any cause (9), which speaks to the widespread systemic effects of CKD.

1.2 Epidemiology of End-stage Renal Disease – National and International Trends

End-stage renal disease (ESRD) is an established chronic kidney disease of growing concern in both Canada and worldwide. In 2009 alone, 5,375 individuals in Canada were newly diagnosed with this disease, an increase of 12% since 2000, and 58% when compared to 1999 (Treatment of End-Stage Organ Failure in Canada, 2000-2009, Canadian Institute for Health Information: 2011 CORR Annual Report). In the United States, although incident rates of ESRD declined in 2010, the overall prevalence of ESRD continues to rise (3). PD is used in conjunction with HD with rates of PD comprising 0-70% of national dialysis programs (10). Over the last decade in Canada, PD modality was selected for 20% of new dialysis patients (2011 CORR Annual Report). In the US, while HD continues to be the treatment modality of choice, rates of peritoneal dialysis increased for the second year in a row, now 6.6% of total RRT (3). The largest growing population of ESRD patients worldwide can be found in Asia, particularly mainland China where rates of diabetes and hypertension are reaching epidemic

proportions. In some Asian countries, the prevalence of ESRD is growing at a staggering 10% annually (11). In addition, PD tends to be much less expensive than HD, in the order of tens of thousands of dollars per patient-year (12, 13). PD has therefore gained increasing preference in developing countries with limited healthcare budget, healthcare infrastructure, and access to health services (14, 15).

1.3 Treatment Modalities for End-stage Renal Disease

As CKD progresses, renal function is no longer adequate and patients eventually require a method of renal replacement therapy (RRT). This ESRD is only encountered by 2% of all patients with CKD (16) yet carries significantly higher health care costs, morbidity, and mortality than any other stage of CKD (3). Patients with ESRD have two options for RRT: kidney transplant and dialysis. Given the overwhelmingly favorable survival, cost, and quality of life indexes, renal transplant is viewed as the preferred treatment modality for ESRD (17-19). However, transplantation is often not available due to the short supply of donor organs and the poor overall health of some ESRD patients, which precludes them as suitable recipients. ESRD patients are therefore required to explore dialysis as either a bridge to eventual transplantation or as a destination treatment modality for ESRD.

The two forms of dialysis currently available are hemodialysis (HD) and peritoneal dialysis (PD). Epidemiological data has demonstrated non-inferior outcomes for PD patients compared to their HD counterparts (20, 21). HD uses a large machine to clean a patient's blood through a vascular circuit, and PD which uses the patient's own abdominal lining – *peritoneal membrane* - as a filter for waste excretion (**Figure 1.1**). HD is usually performed three times weekly in a dialysis facility under the supervision of

doctors and nurses for 3-4 hours, whereby the patient is connected to a dialysis machine through a vascular circuit. In contrast, after receiving training by dialysis facility staff, patients administer PD multiple times daily at home; PD solution (~2L) is instilled through a PD catheter into the peritoneal cavity and 'dwells' for 3-4 hours. Subsequently, the wasteful dialysate is drained from the peritoneum and a fresh bag of PD solution is inserted again (**Figure 1.1**). This process is repeated four times daily everyday by the patient or caregiver without requiring transport to a dialysis facility. It does however require the regular upkeep and maintenance of an indwelling PD catheter and numerous bags of PD solution and other supplies. Summary of these treatment modalities can be found in **Table 1.2**.

	Hemodialysis (HD)	Peritoneal Dialysis (PD)	Renal Transplant
Primary Renal Replacement Mechanism	Hemodialysis machine with filter	Peritoneal membrane and PD solution	Transplanted kidney
Access	Tunneled venous catheter or arterio-venous fistula/graft	Tunneled catheter into peritoneal cavity	N/A
Frequency of renal replacement	Three times weekly; 3-4 hours per visit	Four to Six PD exchanges every day	Continuous
Hospital Contact	Every HD visit	Regular Follow-up every 1-3 months	Regular Follow-up every 1-6 months
Available home-based therapy	Home HD available but few qualify	Yes – almost entirely home based	Yes
Immunotherapy required	No	No	Yes
Common complications	Hypotension, bleeding, catheter infection, cramps, large changes in volume status	Peritonitis, catheter infection, hyperglycemia	Opportunistic infections, increased incidence of malignancy, medication side effects
Cost per patient-year (2012 USRDS)	$87,561	$66,751	$32,914

Table 1.2 Summary of treatment modalities for end-stage renal disease

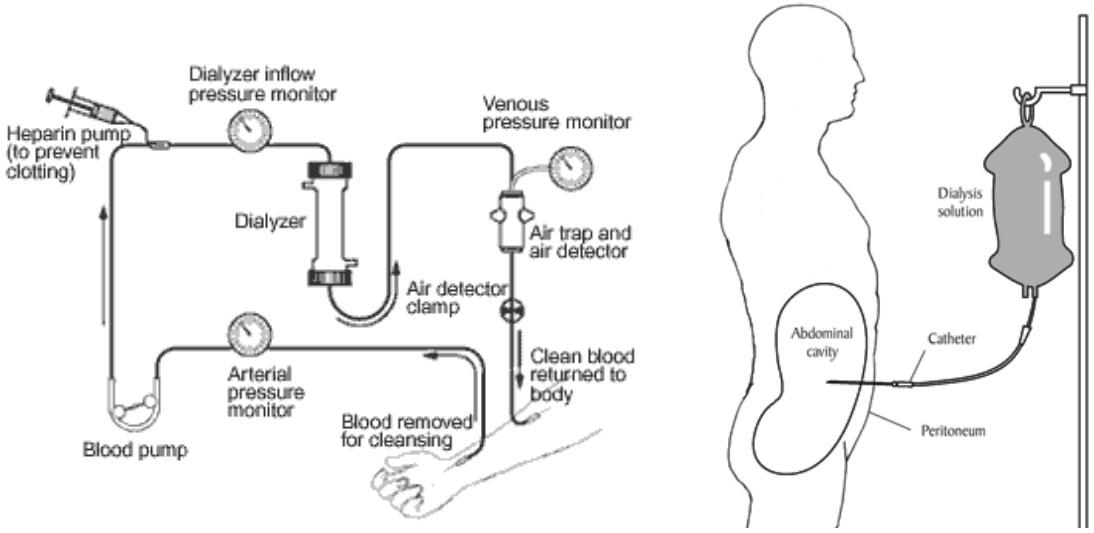

Figure 1.1 Schematic representations of hemodialysis (left) and peritoneal dialysis (right)

1.4 Advantages of Peritoneal Dialysis

The increased autonomy of PD has translated into increased quality-of-life and therapy-satisfaction scores for PD patients when compared HD patients (22, 23). Furthermore, many studies have demonstrated better preserved residual renal function in PD patients (24-26). This directly translates into better handling of phosphate, salt, and fluid, which allows less dietary restrictions for PD patients (27). Patients also demonstrate reduced incidence of anemia and left ventricular hypertrophy (27). This may explain why the incidence of heart failure hospitalization is reduced in PD patients compared with matched HD counterparts (28).

Moreover, there is increasing evidence that PD is a more suitable bridge to renal transplantation than HD for patients with ESRD. Because they are not being exposed to a vascular circuit, patients on PD will have lower incidence of hepatitis B or C infection and thus fewer complications with subsequent immunosuppressive therapy (29). Transplanted graft outcomes appear to be improved with PD patients compared to

matched HD controls (30-33). Patients on PD who are naïve to HD will have preserved vascular access for future dialysis in the event of graft failure. Therefore, there is an incentive to initiate PD first and attempt to offer PD as the exclusive pre-transplant dialysis modality for adult and pediatric patients awaiting timely renal transplant.

1.5 Peritoneal Dialysis – Physiology of the Three Pore Model (TPM)

1.5.1 Distribution of Pores

The central mechanism of action of PD and its ability to remove solutes and water from the bloodstream is derived from the Three Pore Model (TPM) of PD. First introduced by Rippe et al in 1991 (34), it has been reviewed most recently in 2004 (35) and has contributed significantly to our understanding of the mathematical characterization of peritoneal membrane failure (36).

The bloodstream in separated from the peritoneal cavity by a semi-permeable peritoneal membrane that consists of three pores of varying sizes and physical properties. The overwhelming majority of pores (~90%) are called **small pores** of radius 43Å that are permeable to both fluid and small solutes but impermeable to macromolecules (i.e., proteins). **Large pores,** accounting for 8% of total pore area, have a radius of 250Å; they are non-selective and allow for free movement of water, solutes and macromolecules such as proteins. **Ultra-small pores – also known as aquaporins (AQP1) –** account for only 2% of total pore area and radius 4Å; they function as a water-selective channel and reject the transport of any solutes or macromolecules (37) (**Figure 1.2)**.

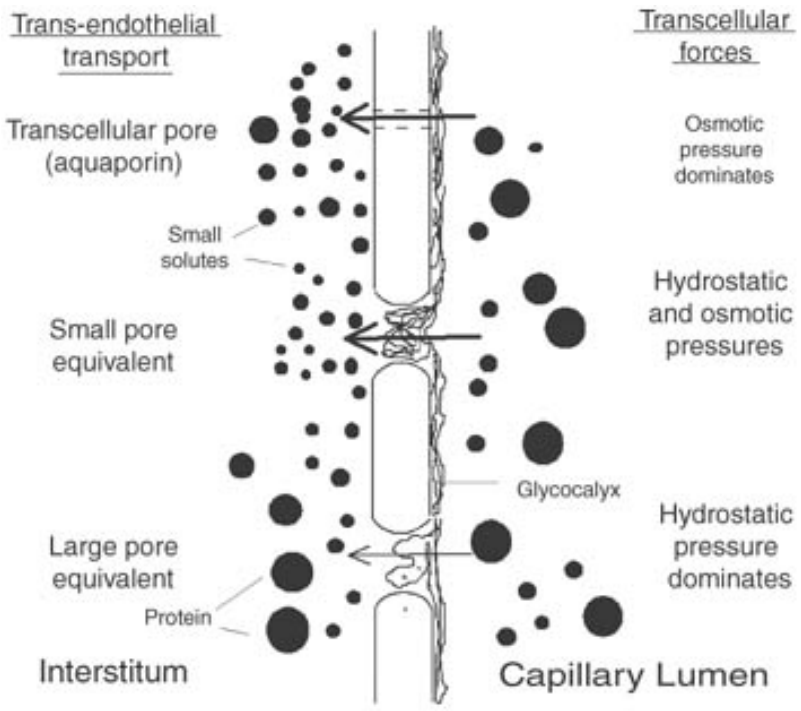

Figure 1.2 Schematic representation of the three-pore model
The peritoneal membrane forms the semi-permeable barrier that separates the peritoneal capillaries from the peritoneal cavity. It consists of three pores of varying sizes: **large pores** are permeable to water, small solutes (i.e. anion, cations, small molecules), and macromolecules (i.e. proteins). **Small pores** are permeable to water and small solutes. **Ultrasmall pores**, also known as **aquaporins** are permeable only to water. Reproduced with permission from Flessner et. al (38).

1.5.2 Osmotic Agents, and Ultrafiltration

Water transport in PD, which is termed **Ultrafiltration (UF)**, occurs across the peritoneal membrane via an osmotic pressure gradient. UF and osmotic pressure are equally dependent on both the size and concentration of the osmotic agent (e.g., glucose, icodextrin, etc.). Osmotic agents are characterized by their **reflection coefficient (σ)**, which defines the ability of the peritoneal membrane to reject the particular molecule of interest. Larger solutes have a higher reflection coefficient and will remain inside the peritoneum for longer; these molecules usually remove more fluid *per unit solute* compared to small molecules. Yet, large solutes achieve less maximal concentration in

solution. Small solutes can achieve higher concentration in solution and therefore a larger osmotic gradient, but their small size allows for rapid systemic absorption and rapid loss of this gradient - the osmolality of the dialysate - over the course of the PD dwell. Examples of reflection coefficients of different osmotic agents have been calculated (39) and can be found in **Table 1.3**. Osmotic agents exist as molar concentrations in solution, and their ability to generate osmotic pressure is modified by their reflection coefficients.

	Molecular Weight (Da)	Molecular Radius (Å)	Reflection Coefficient (σ)
NaCl	58.5	2.3	0.030
Glucose	180	3.66	0.045
Glycerol	92	2.7	0.034
Sorbitol	122	3.0	0.037
Amino Acids	75-214	2.4-4.0	0.031-0.049
Glucose polymer	5,000	13-14	0.29
	10,000	~17	0.40
	20,000	~23	0.60
Neutral dextran	70,000	50	~0.9
Albumin	69,000	36	~0.9
Polypeptides	800-1000	6-8	~0.12

Table 1.3 Reflection coefficients (σ) for various osmotic agents in peritoneal dialysis
Reflections coefficients range between 0-1. Values close to zero represent free movement of the osmotic agent across the peritoneal membrane whereas values close to one represent impermeability of the osmotic agent. Adapted from Rippe et al (39).

1.5.3 Free Water Transport

Free water transport (FWT) is defined as the movement of water through the aquaporins without concomitant movement of solute. FWT can be simulated via computer modeling (37) or measured empirically (40-43). The phenomenon of *sodium sieving* is a drop in initial dialysate sodium concentration during the first few hours of the PD dwell and correlates with "water only" movement across aquaporins that dilutes the sodium in PD solution. Sodium sieving is observed with glucose-based UF, but is absent with

icodextrin UF when the movement of water through small pores is accompanied by sodium (37) (**Figure 1.3**).

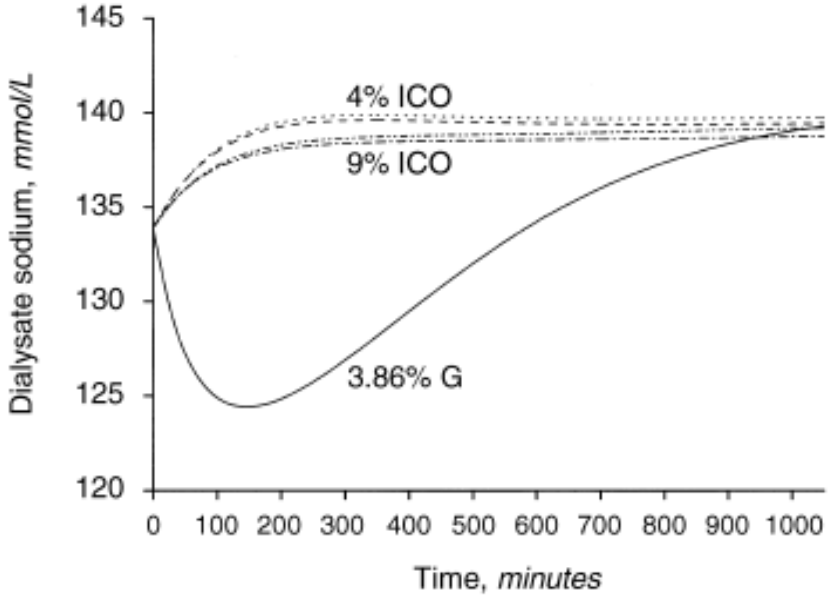

Figure 1.3 Sodium sieving in peritoneal dialysis
Dialysate sodium concentrations (mmol/L) during peritoneal are simulated using computer modeling for hypertonic glucose (3.86% G) and icodextrin (ICO). Reproduced with permission from Rippe et al (37).

Although aquaporins comprise only 2% of the total pore area, they account for 40-50% of the water transport in glucose-based PD therapy (35, 44). Larger osmotic agents such as icodextrin usually maintain a low level of FWT of approximately 5-10% (40). The disconnect between aquaporin area and %FWT of glucose-based PD is attributed to the highly selective nature of aquaporins and a theoretical reflection coefficient of 1.0 across these ultra-small pores, even for small solutes such as glucose. The difference in pore-specific reflection coefficients between small pores and aquaporins is much greater for glucose (0.049 vs. 1.0) than for larger osmotic agents such as icodextrin (0.767 vs. 1.0) (35, 37). This correlates with a markedly higher osmotic pressure across the aquaporins versus small pores. For larger osmotic agents, there is a smaller pressure discrepancy

between aquaporins and small pores and thus very little diversion of water transport through aquaporins.

1.5.4 Solute Transport

Solutes such as urea, sodium, and potassium are transported across the peritoneal membrane by diffusion down a concentration gradient or are coupled with ultrafiltration through a convective process termed *solvent drag* (36). The ability of the peritoneal membrane to transport solutes can be measured by calculating the **dialysate-to-plasma ratio (D/P ratio)** of a given solute at any time point during the PD dwell. Solute transport is described as higher if the value approaches unity (equilibrium) earlier in the PD dwell. While higher solute transport status may conceivably be viewed as more efficient and advantageous, this is often not the case. Because there is such a high concentration gradient for uremic toxins and potassium to diffuse from the bloodstream into the peritoneal fluid, adequate removal of these substances rarely poses a challenge during PD therapy. However, higher transport status also correlates with increased absorption of glucose from the peritoneal cavity into the bloodstream. This leads to loss of osmotic gradient, and inadequate fluid removal during PD. Longitudinal studies document increasing transport status of PD patients over time, which has been correlated with increasing glucose exposure and multiple episodes of peritonitis (45-47). Ultimately, high transport status correlates with increased mortality for PD patients (48).

1.6 Challenges with PD Therapy

The current conventional PD solution is prepared using a high concentration of glucose as a primary osmotic agent. This glucose produces systemic and locoregional health complications for PD patients. Daily exposure to glucose can cause hyperglycemia,

hyperinsulinemia, obesity and exacerbation of Diabetes (49). The estimated daily glucose load from PD is approximately 100-300g (50). Moreover, exposure to glucose and glucose-degradation-products (GDPs) has been shown to directly damage the peritoneal membrane leading to **Ultrafiltration Failure (UFF)** (36, 51-54). This phenomenon is characterized clinically by increased membrane permeability to small solutes, rapid absorption of intraperitoneal glucose, and inadequate fluid removal during PD. UFF, and thus inadequate fluid removal with PD, is the second leading cause (behind peritonitis) of technique failure requiring cessation of PD therapy and transition to HD (13). Recently it has been suggested that the use of glucose may be associated with increasing susceptibility of PD patients to the development of peritonitis due to the culture medium that glucose provides for bacterial replication (51); this peritonitis further promotes peritoneal membrane failure through damage and fibrosis. Reducing peritoneal inflammation will delay UFF and prolong the time patients spend on PD. This will improve patient quality-of-life and also limit the healthcare expenses required to transition and maintain patients on HD. Minimizing glucose exposure, therefore, is expected to prevent some of the metabolic complications associated with PD. Also, improving locoregional host defense and reducing the glucose concentration in the peritoneum may also lead to declining rates of aseptic and bacterial peritonitis.

1.7 Evaluation of Peritoneal Membrane Biocompatibility

The peritoneal membrane experiences significant changes with prolonged exposure to PD solution. While clinically this corresponds with UFF, these changes can also be quantified structurally or with biochemical techniques. Peritoneal membrane capillaries will undergo reduplication of the basement membrane and fibrotic expansion of the

submesothelial compact zone; these capillaries exhibit a structure that mimics diabetic microangiopathy (55-58). Furthermore, maladaptive angiogenesis and neoproliferation of peritoneal capillaries corresponds with the increased solute transport that is observed in PD patients; this is thought to be mediated in large part by vascular endothelial growth factor (VEGF) and can be detected in dialysate effluent (59, 60). Immunohistochemical staining of peritoneal membrane in human PD patients and rodent models demonstrates increased expression of advanced glycosylation end-products (AGEs) (61-63). Taken together, these findings further implicate glucotoxicity in the pathophysiology of peritoneal membrane failure.

The human peritoneal mesothelial cell (HPMC) can be viewed as the basic functional unit of the peritoneal membrane and has been studied extensively in peritoneal dialysis both *in vivo* and with *ex vivo* culturing techniques (64). Viability assays of cultured HPMCs have often been used as a surrogate for *in vivo* biocompatibility of PD solutions (65-69). These cells secrete cancer antigen 125 (CA-125) and levels of CA-125 in PD effluent seem to correlate with an increased total mesothelial cell mass (70). CA-125 levels decline longitudinally in PD patients (71) and thus, higher levels may serve as a useful biomarker for peritoneal membrane integrity (72).

One of the central pathways involved in peritoneal membrane failure relates to the **epithelial-to-mesenchymal transition (EMT)** of HPMCs (53, 54, 73). Mesothelial cells undergo a phenotypic switch that corresponds to the increased secretion of VEGF, collagen, cyclooxygenase-2, and snail transcription factors; loss of epithelial architecture is associated with downregulation of E-cadherin, cytokeratins, and desmoplakin. EMT also involves the proliferation of fibroblasts that contribute to interstitial fibrosis (53).

EMT has been linked with the transforming growth factor beta (TGF-β) pathway (74) and indeed, TGFβ antagonists have blocked the EMT observed in HPMCs after exposure to PD solutions (75, 76).

Ultimately none of these biomarkers have been routinely adopted for clinical use. Although many surrogates for biocompatibility have been explored as outlined above, the true benchmark for peritoneal membrane biocompatibility should still be viewed as the clinical end-point of technique survival (i.e. time on PD therapy) (51). To this end, there has been a paucity of studies that have made the connection between bench and bedside.

1.8 Alternative Osmotic Agents

There has been ongoing research targeted to improve the biocompatibility of PD solutions. Icodextrin is a large glucose-based polymer (mW ~17 kDa) designed to mitigate many of the problems encountered from long-term glucose exposure. Indeed, clinical trials have shown improved metabolic parameters in patients prescribed PD regimens containing icodextrin (50), despite the elevated levels of blood maltose seen with icodextrin therapy. Notably, cell count in the peritoneal effluent of PD patients is significantly higher with icodextrin than with glucose (77), indicating the potential ongoing role of icodextrin in peritoneal inflammation. The main clinical role of icodextrin has been in patients with established UFF when glucose can no longer remove water from the body. Due its large size, icodextrin will remain intraperitoneal for longer and therefore achieve more reliable ultrafiltration compared to glucose. Moreover, due to the relatively slow fluid kinetics of icodextrin, maintaining adequate 24-hr fluid removal would be challenging on a daily basis; it is therefore unlikely that icodextrin will completely replace glucose as the sole osmotic agent in PD. Even as twice-daily

icodextrin prescription is currently being investigated experimentally, it has yet to be approved by for widespread use by national health licensing authorities (78).

L-carnitine has also been tested in pre-clinical rodent models of PD and a small group of PD patients (79). It has been shown as an effective substitute for glucose as an osmotic agent with dose-dependent ultrafiltration and improved viability of murine fibroblasts and human endothelial cells. Although promising, this therapy has not received widespread adoption, likely due to the absence of larger clinical trials, and the relative similarity between L-carnitine and glucose with respect to molecular size and weight (80). Similarly, glycerol monomer PD solutions have been tested safely in small clinical trials but have also not broken into mainstream PD therapy. This may be attributed to the similarity with glucose, or potentially from reports of lipid derangement and hyperosmolar syndrome (81, 82).

In an effort to offer nutritional support to PD patients already experiencing protein-energy malnutrition and also avoid glucose exposure, amino acids have been explored as a potential osmotic agent. Nutrineal™ is a 1.1% amino acid PD solution that can substitute one of the four daily PD exchanges, and does offer effective ultrafiltration (83). However, studies are inconsistent as to whether this amino acid support actually improves nutritional status in these PD patients (84). Moreover, the nitrogen load from the amino acids has been shown to increase blood urea levels and exacerbate metabolic acidosis (85). Therefore, there is still need for ongoing research into alternative biocompatible PD solutions that can be used multiple times daily.

1.9 HPG as Candidate Osmotic Agent in PD

Hyperbranched polyglycerol (HPG) is a branched compact highly water soluble polyether polymer (**Figure 1.4**), synthesized by a single step multi-branching ring opening polymerization of glycidol under slow monomer addition (86).

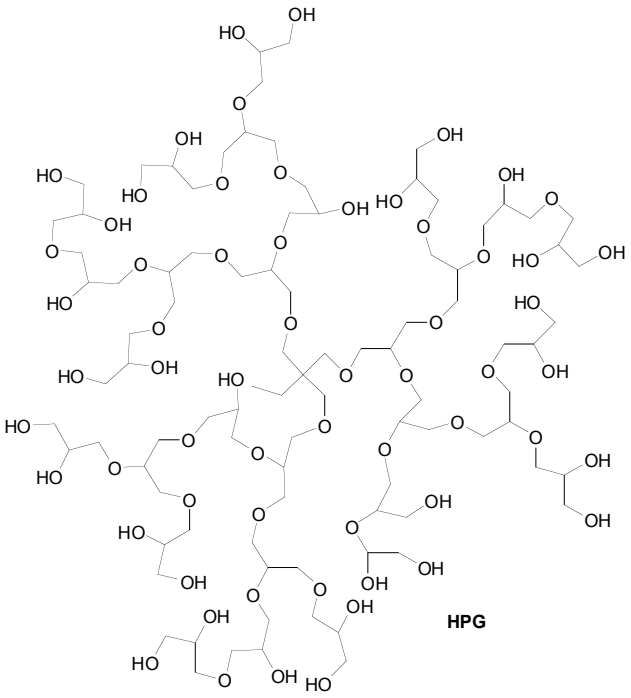

Figure 1.4 Chemical structure of hyperbranched polyglycerol (HPG)

HPG has been investigated for its potential in many biomedical applications, including as a substitute for serum albumin (87, 88) neutralizing agents for heparin (89) anti-inflammatory agents (90), and drug delivery (91-94). In collaboration with the Kizhakkedathu laboratory, we began investigating the use of HPG as a substitute for glucose-based osmotic agents in peritoneal dialysis. Many experimental studies have demonstrated that HPG or its derivatives are highly blood compatible, non-immunogenic and nontoxic molecule (87, 95-97). HPG does not activate the platelet, coagulation or complement systems (87). Unlike other polymers, HPG has been shown very limited organ accumulation after intravenous injection (87, 98). We hypothesized that HPG

would demonstrate good application as an osmotic agent for peritoneal dialysis based on the following chemical properties:

- HPG can be synthesized with very good control over a wide range of molecular weights; this allows HPG to be modified to optimize its ultrafiltration capacity.
- Avoiding glucose or glucose-based polymers will reduce metabolic complications of hyperglycemia and exacerbation of diabetes, which are exceedingly common in patients with ESRD.
- The multiple hydroxyl groups on HPG function as a buffer to maintain physiologic pH for PD solution.
- HPG PD solutions are significantly less viscous than icodextin PD solutions owing to the very low intrinsic viscosity in water in the order 4 to 7 cc g^{-1}. HPG does not precipitate proteins at high concentrations unlike polyethylene glycols and does not adsorb to cells or aggregate cells even at high concentrations (99). More polymer can therefore be dissolved in solution to achieve maximal ultrafiltration capacity.
- Systemic toxicity of the polymer can potentially be mitigated in the future if necessary by modifying the functional groups on HPG.
- Glycidol, the monomer used for the synthesis of HPG, can be easily synthesized from glycerol - a by-product of biorefining. The process of HPG synthesis is scalable for industrial-level production.

Taken together, these properties make HPG a promising candidate for use in PD. The goal of this thesis is to test HPG as a glucose-sparing osmotic agent in a rodent model of PD.

2. Materials and Methods

2.1 Animal Care

Male inbred Sprague-Dawley rats (~300 g bodyweight, 10-12 weeks old) were purchased from the Charles River Laboratories International, Inc. (Wilmington, MA, USA), and maintained in the animal facility of the Jack Bell Research Centre of the University of British Columbia (Vancouver, British Columbia, Canada). Animal experiments were performed in accordance with the Canadian Council on Animal Care guidelines under protocols approved by the Animal use Subcommittee at the University of British Columbia (protocol #A10-0344)

2.2 Preparation of HPG Solutions

HPG (Mn = 500 g/mol, 1000 g/mol, and 3000 g/mol, Mw/Mn = 1.1, where Mn was the number of average molecular weight, Mw represented the weight of average molecular weight, and Mw/Mn indicates polydispersity index (PDI)) was synthesized and characterized as described previously (86, 100). The absolute molecular weight and PDI of the HPG was determined by gel permeation chromatography (GPC) using a DAWN-EOS multi-angle laser light scattering (MALLS) (Wyatt Technology Inc.) and Optilab RI detectors in aqueous 0.1N $NaNO_3$ solution. The experimentally determined dn/dc value 0.120 mL/g was used for the molecular weight determination. Ultrahydrogel columns (Waters Corp., USA) were used for the analysis.

For the first round of experiment, HPG solutions with concentrations 2.5, 5, 7.5 and 15 wt% were prepared by dissolving HPG (2.5-15 grams of 3 kDa HPG) in 100 mL of a sterile electrolyte solution containing sodium chloride (NaCl, 5.38 g/L), sodium lactate ($NaC_3H_5O_3$, 4.48 g/L), calcium chloride ($CaCl_2$ $2H_2O$, 0.183 g/L) and magnesium

chloride ($MgCl_2$ $6H_2O$, 0.051 g/L), the same electrolyte composition as in control Dianeal® (2.5%, PD4 CAPD solution, Baxter Healthcare Co, Deerfield, IL, USA) (PDS). The osmolality of each solution was measured using Advanced® Model 3320 Micro-Osmometer (Advanced Instruments, Inc., Norwood, MA, USA) in the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada), and pH of each HPG solution were recorded using a laboratory pH meter in a period of 10 min.

For the second round of experiments, the electrolyte composition of HPG solution has been modified to simulate the lactate/bicarbonate buffer system of our new Physioneal™ control fluid (Physioneal™ 2.27% PD4 CAPD solution, Baxter Healthcare Co.) (PYS). HPG solutions were prepared by dissolving the concentration of HPG polymer (4.8% of 0.5 kDa, 6.0% of 1 kDa, 14% of 3 kDa) in 100 mL of sterile water containing the following electrolytes: sodium chloride (NaCl, 5.38 g/L), sodium lactate ($NaC_3H_5O_3$, 4.48 g/L), calcium chloride ($CaCl_2$ $2H_2O$, 0.183 g/L), sodium bicarbonate ($NaHCO_3$, 2.1 g/L), and magnesium chloride ($MgCl_2$ $6H_2O$, 0.051 g/L). The osmolality of each solution was measured using the Micro-Osmometer (Advanced Instruments, Inc., Norwood, MA, USA) in the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada).

2.3 Rodent Model of PD

The efficacy and biocompatibility of HPG solutions versus control PDS or PYS was examined with Sprague-Dawley rats, a preclinical model of PD. Under general anesthesia, 30 mL of pre-warmed HPG solutions, or PDS or PYS was slowly injected into the peritoneal cavity. Immediately (basal control at 0 hour) or at time points 0.5, 2, 4, and 8 hours following intraperitoneal injection, animals were sacrificed and peritoneal

effluent and serum were collected. The first round of experiments comprised only the 4-hour time point; the second set of experiments included all different time points from 0.5 to 8 h. The parietal peritoneum was collected in three randomly selected rats from each group at the 4 hr time point. We used a single intraperitoneal injection in order to avoid the confounding factor of catheter insertion that might exacerbate peritoneal injury (101).

2.4 Evaluation of Ultrafiltration and Waste Removal

As a marker of ultrafiltration capacity, the volume of peritoneal effluent recovered after intraperitoneal injection was compared to the volume recovered at 0 hour (102). We used urea nitrogen as a marker of waste substance because the creatinine levels in peritoneal effluents were below the minimal level for measurement in the laboratory. Urea values were measured from both blood and serum in the Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC, Canada). Absolute peritoneal urea removal was calculated by multiplying the urea concentration in recovered dialysate (D) with its volume (V). Small solute transport was evaluated at multiple time points by calculating D/P ratio of urea, where P represents the urea concentration in plasma**.** Urea clearance was calculated by multiplying the D/P ratio with the volume of the dialysate (D/P × V). Similar to previous studies, uremia was not considered necessary in our rodents to accurately assess fluid and waste transport over one dwell (103).

2.5 Evaluation of Free Water Transport

FWT was calculated using the La Milia method (42) that has also been applied to rodent models in previous studies (43). The equations can be found in **Appendix** and are derived from sodium and UF values obtained at 2h from recovered dialysate and blood as measured in Vancouver Coastal Health Regional Laboratory Medicine (Vancouver, BC,

Canada). Although FWT is ideally calculated as early as possible in the dialysis dwell, we found the UF values at 0.5 hour to be too small (less than 3 mL) and thus resulted in large error ranges and uninterpretable FWT results. Sodium values for initial dialysate were measured directly as 138 mmol/L for PYS, 132 mmol/L for 0.5 HPG, and 130 mmol/L for 1 kDa and 3 kDa HPG; initial volume of dialysate is 30 mL. FWT is reported as a percent of total UF.

Sodium sieving is defined as the early drop in dialysate sodium concentration as it is diluted by aquaporin-mediated FWT; this is a good approximation of aquaporin function (104) and demonstrates a high correlation with other calculated methods of FWT (105). Sodium sieving was reported as a percent drop from initial dialysate sodium concentrations (mmol/L).

2.6 Evaluation of Sodium Removal

Adequate sodium removal impacts volume status management for PD patients and directly correlates with improved survival (106). Total sodium removal (NaR) at any time point *t* by PD solution was calculated as $Na_t - Na_{initial}$ whereby $Na = Volume_{dialysate} \times [Na_{dialysate}]$. Initial dialysate volumes were set as 30 mL and sodium values for initial dialysate were measured directly as 138 mmol/L for PYS, 132 mmol/L for 0.5 HPG, and 130 mmol/L for 1 kDa and 3 kDa HPG.

2.7 Histological Examination

Peritoneal tissues were fixed in 10% neutral buffered formalin, followed by embedded in paraffin wax. Sections were cut at 4-μm thickness and stained with hematoxylin and eosin (H&E). Specimens were examined under microscopic view (×400 magnification) in two separate sections of each strip of the peritoneum in a blinded fashion. Submesothelial

thickness was measured and polymorphonuclear infiltration was used as a marker of peritoneal inflammation.

For the second round of experiment, the H&E-stained sections were scanned with Leica SCN400 Slide scanner (Leica Microsystens Inc., Concord, ON, Canada), and the measurement of submesothelial thickness for each animal was performed in at least 20 non-overlapping and randomly selected sites in two serial sections using the Digital Image Hub Slidepath Software Solution (Leica Microsystems Inc.). The presence of polymorphonuclear infiltration in the histological analyses was used as a marker of peritoneal inflammation.

2.8 Flow Cytometric Analysis

The presence of detached mesothelial cells and neutrophils in the effluents was assessed with flow cytometric analysis as an *in vivo* biocompatibility profile of PD solutions after PD exposure. Flow cytometric analysis was performed on a BD FACSCanto™ II (BD Biosciences, Mississauga, ON, Canada). At least 10,000 events were counted for each sample, and data were analyzed with FlowJo software (Tree Star, Ashland, OR). Neutrophils/granulocytes, lymphocytes, and monocytes in PD effluent were identified based on their size and granularity in the dot plot of forward scatter (FSC) versus side scatter (SSC) as described previously (107), and were counted in the gated area as the percentage of total cell count in the peritoneal effluents.

Mesothelial cell (MCs) detachment into peritoneal dialysis effluent is a reliable marker for peritoneal membrane injury (64, 108, 109). MCs express a unique cell surface protein, human bone marrow endothelial cell (HBME)-1 (110) that was identified by fluorescence-activated cell sorting (FACS) with rabbit polyclonal anti-HBME-1 antibody

conjugated fluorescein isothiocyanate (FITC) (anti-HBME-1-FITC, Biorbyt LtD, Riverside, UK) versus rabbit polyclonal anti-mouse IgA-FITC (Cayman Chemical, Ann Arbor, MI, USA) as a staining control. The cells without antibody stain were used as a negative background for FITC positivity.

2.9 May-Grünwald-Giemsa (MGG) Cytochemical Stain

The presence of MCs in the peritoneal effluents was confirmed with MGG cytochemical stain as previously described (109, 111). In brief, cells in the peritoneal effluents were spun down by the centrifugation at 6,000 rpm for 10 min, and were smeared over microscope glass slides. After fully air-dried, cell smears were fixed in methanol. Following rehydration with PBS, the cell smears were stained with May-Grünwald solution (Sigma-Aldrich Canada, Oakville, ON, Canada) (1:5 dilution with PBS) for 10-15 min, washed with PBS and then stained again with Giemsa stain solution (Sigma-Aldrich Canada) (1:5 dilution with PBS) for 30 min. The color of different types of cells was differentiated by a further wash with PBS.

2.10 Statistical Analysis

Data were presented as mean ± standard derivation (SD) of each group. Two-tailed Student's *t*-test or analysis of variance (ANOVA) using Prism GraphPad software (GraphPad Software, Inc., La Jolla, CA, USA) was used as appropriate for data analyses. A p-value of ≤ 0.05 was considered significant.

3. Proof of Concept of HPG as Effective Osmotic Agent in PD

3.1 Introduction

Novel osmotic agents must fulfill three criteria in order to be considered for use in PD:

1. They must produce net fluid movement into the peritoneal cavity (ultrafiltration)
2. They must facilitate removal of waste molecules from the bloodstream into dialysis fluid (clearance)
3. They must cause the least amount of mesothelial injury or inflammation of the peritoneal membrane (biocompatibility).

To evaluate these criteria in a proof of concept study, 3 kDa HPG solutions at various concentrations (**Table 3.1**) were compared directly with conventional glucose-based PD solution (Dianeal® 2.5% PD4 CAPD solution, Baxter Healthcare Co). These experiments consisted of one time point four hours after intraperitoneal injection.

PD Solution	Osmolality (mOsm/kg)	pH
2.5% 3 kDa HPG	279	6.62 (6.60 - 6.67)
5% 3 kDa HPG	294	6.79 (6.63 - 6.90)
7.5% 3 kDa HPG	324	7.19 (7.04 – 7.30)
15% 3 kDa HPG	424	7.27 (7.19 – 7.32)
2.5% Dianeal™	396*	5.2 (4.0 – 6.5)

Table 3.1 Biochemical profiles of 3 kDa HPG solutions and Dianeal
Osmolality for HPG solutions was measured in the Vancouver Coastal Health Regional Laboratory and pH was calculated using a laboratory pH meter in a period of 10 min. Values for Dianeal™ were taken from the Baxter product catalog. (http://www.baxter.com/downloads/patients_and_caregivers/products/dianeal_ambulexpd 2.pdf). * indicates calculated osmolarity (mOsm/L) instead of measured osmolality (mOsm/kg).

3.2 Ultrafiltration Profile

Figure 3.1 describes the ultrafiltration capacity of varying concentrations of 3 kDa HPG and PDS 4 hours after intraperitoneal injection. HPG demonstrates a concentration/osmolality-dependent increase in the volume of recovered peritoneal effluent, 40 ± 1.24 mL by 7.5% HPG ($p < 0.0001$, vs. 0 hour) and 43.33 ± 5.24 mL by 15% HPG ($p < 0.0001$, vs. 0 hour). Peritoneal effluent volume by 5% HPG solution (27.88 ± 1.65 mL) after 4 hours was not statistically different from 0 hour ($p = 0.7371$). The ultrafiltration by 7.5% HPG solution was similar to 37.23 ± 4.72 mL by PDS after 4 hours of dwell ($p = 0.1879$), but 15% HPG achieved more effective ultrafiltration than PDS (P = 0.0347). These data in aggregate demonstrate the efficacy of the HPG polymer as an osmotic agent in PD solution.

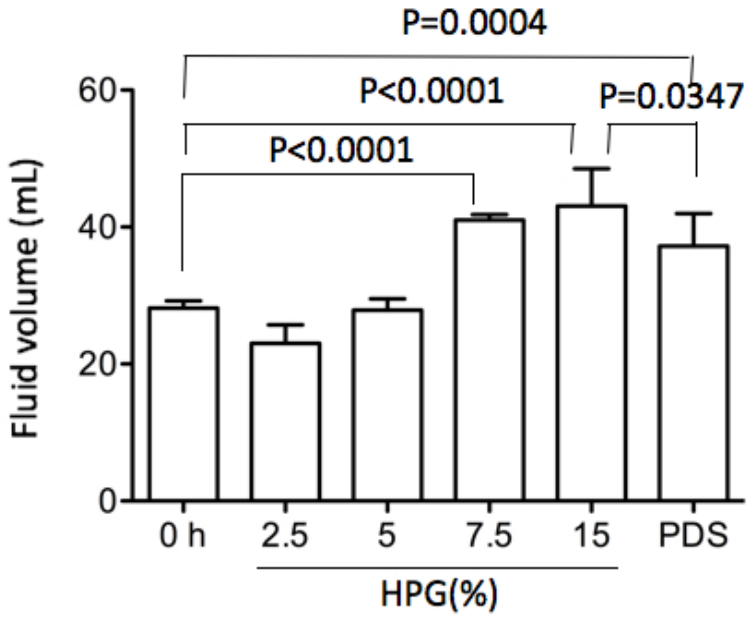

Figure 3.1 Ultrafiltration profiles of HPG solutions
Each rat received intraperitoneal injection of 30 mL of HPG solution and PDS. After 0 or 4 hours, the fluid was recovered from peritoneal cavity, and its volume was measured. Groups: 0 hour control (n = 10), 2.5% HPG (n = 11), 5% HPG (n = 4), 7.5% HPG (n = 4), 15% HPG (n = 6), and PDS (n = 11). Reproduced with permission from Mendelson et al (112).

3.3 Urea Removal

Table 3.2 describes the urea concentration, urea removal, D/P urea ratio**,** and urea clearance of dialysate for the 3 kDa HPG solutions and PDS. Note that both 7.5% and 15% HPG solutions achieved better urea clearance, higher urea concentrations in dialysate, and removed more total urea than PDS after 4 hours of dwell time. D/P urea ratios for 5%, 7.5% and 15% HPG were not statistically different from PDS ($p > 0.05$) indicating similar small solute transport characteristics of these solutions. D/P ratio of 2.5% HPG solution was higher than PDS ($p = 0.0248$) in the context of negative UF from the peritoneal cavity into the bloodstream. Net urea removal and urea clearance of 7.5% HPG was higher than PDS despite similar rates of ultrafiltration (**Figure 3.1)** and solute transport.

Experimental Groups	**Urea Concentration in D (mmol/L)**	**Total Urea in D (mmol)**	**Urea Clearance (mL per 4 h)**	**D/P Ratio of Urea**
2.5% HPG	4.388 ± 0.383	0.101 ± 0.015	22.44 ± 3.75	0.97 ± 0.06
5% HPG	5.575 ± 0.427	0.156 ± 0.02	23.13 ± 3.71	0.94 ± 0.06
7.5% HPG	7.3 ± 1.08	0.3 ± 0.047	36.08 ± 2.05	0.95 ± 0.06
15% HPG	7.85 ± 0.74	0.337 ± 0.042	39.17 ± 5.21	0.91 ± 0.04
PDS	5.356 ± 0.993	0.198 ± 0.043	32.01 ± 2.67	0.88 ± 0.08
p values				
*15% HPG	*0.0002	*0.0001	*0.0037	*0.4384
**7.5% HPG versus PDS	**0.0088	**0.0027	**0.0209	**0.3075

Table 3.2 Urea measurements in dialysate (D) and plasma (P) for 3 kDa HPG
Urea values were measured in dialysate and serum in Vancouver Coastal Health Regional Laboratory (Vancouver, BC, Canada).

3.4 Histological Examination

Histological examination revealed that peritoneal membrane thickness increased after 4 hours for all the treatment groups compared to time 0 hour after exposure to hypertonic solution (7.5% or 15% HPG and PDS solution), and the thickness of the peritoneal membrane of HPG groups was only a half of that in the PDS group (**Figure 3.2**). Moreover, when the polymorphonuclear infiltration (*i.e.* neutrophils) was examined in H&E-stained tissue sections, fewer neutrophils were seen in the peritoneal membrane, particularly the swelling submesothelial zone, of HPG groups as compared to PDS group.

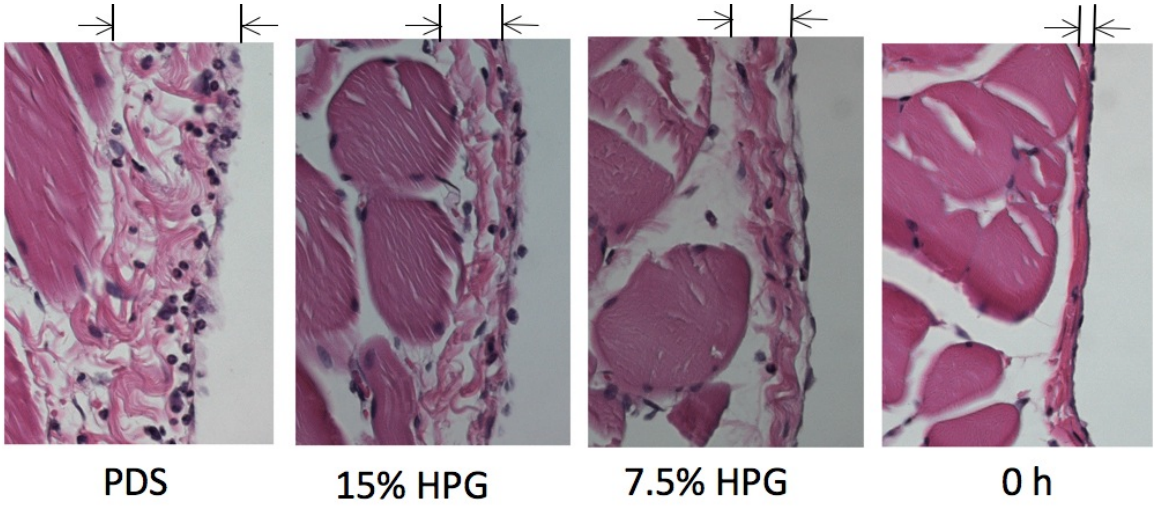

Figure 3.2 HPG solutions demonstrate less peritoneal damage and neutrophil infiltration on histological examination
Peritoneal membrane histology sections taken from rats after 4 hours of PD exposure and control at 0 hour. The tissue sections were stained with H&E. The thickness of the peritoneal membrane was indicated by the distance between two arrows. Cellular infiltrates stained by dark blue: polymorphonuclear leukocytes including neutrophils. Reproduced with permission from Mendelson et al (112).

3.5 Neutrophil Response

The histological observation of reduced neutrophil infiltration was further supported by flow cytometric data shown in **Figure 3.3**. The recovered dialysate from the HPG groups had proportionally less neutrophils than PDS (3.63 ± 0.87% vs. 9.31 ± 2.89%; $p < 0.0001$).

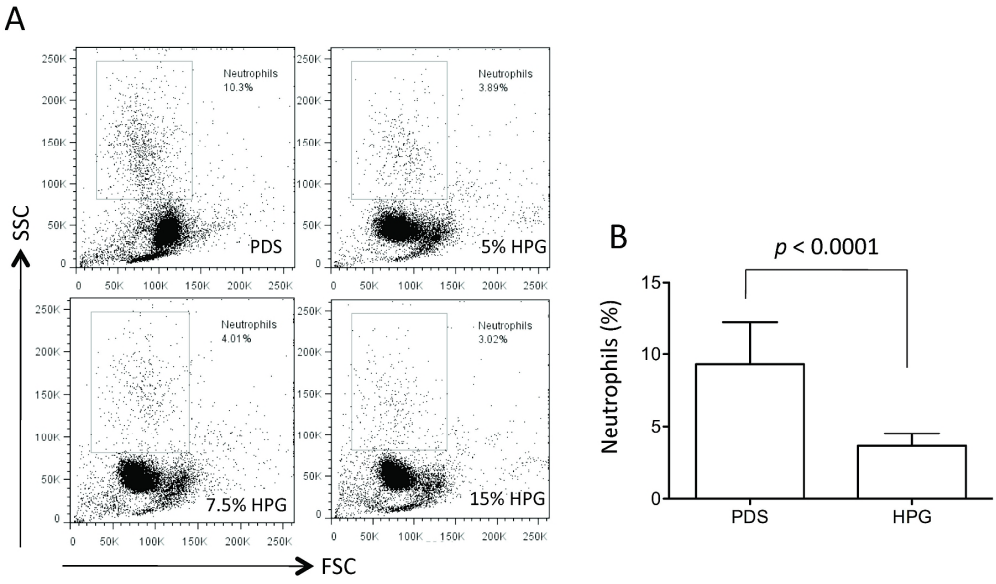

Figure 3.3 HPG solution induces less neutrophil infiltration
The presence of neutrophils in the recovered fluids was used as a biomarker for peritoneal inflammation and was quantitatively determined using a flow cytometry. (A) Data were presented as a typical percentage of neutrophils, rectangled in a SSC/FSC plot of each group. (B) Data were presented as mean ± SD of neutrophils in PDS versus pooled HPG solutions. Reproduced with permission from Mendelson et al (112).

3.6 Mesothelial Cell Detachment

In these experiments, the data were not statistically different between HPG groups based on HPG concentration and therefore HPG data were pooled for analysis. As shown in **Figure 3.4**, HBME-1 positive mesothelial cell detachment did not increase from control stain in effluent of the HPG group (0.70 ± 0.31% vs. 0.36 ± 0.35%; $p = 0.1832$), but was elevated in the PDS group (1.62 ± 0.68% vs. 0.41 ± 0.31%; $p = 0.0031$). The presence of MCs in the peritoneal effluents of PDS group was further confirmed by MGG stain

(Figure 5C), evidenced by the presence of MCs in MGG-stained cell smears of peritoneal effluents from PDS group, but was absent in HPG groups.

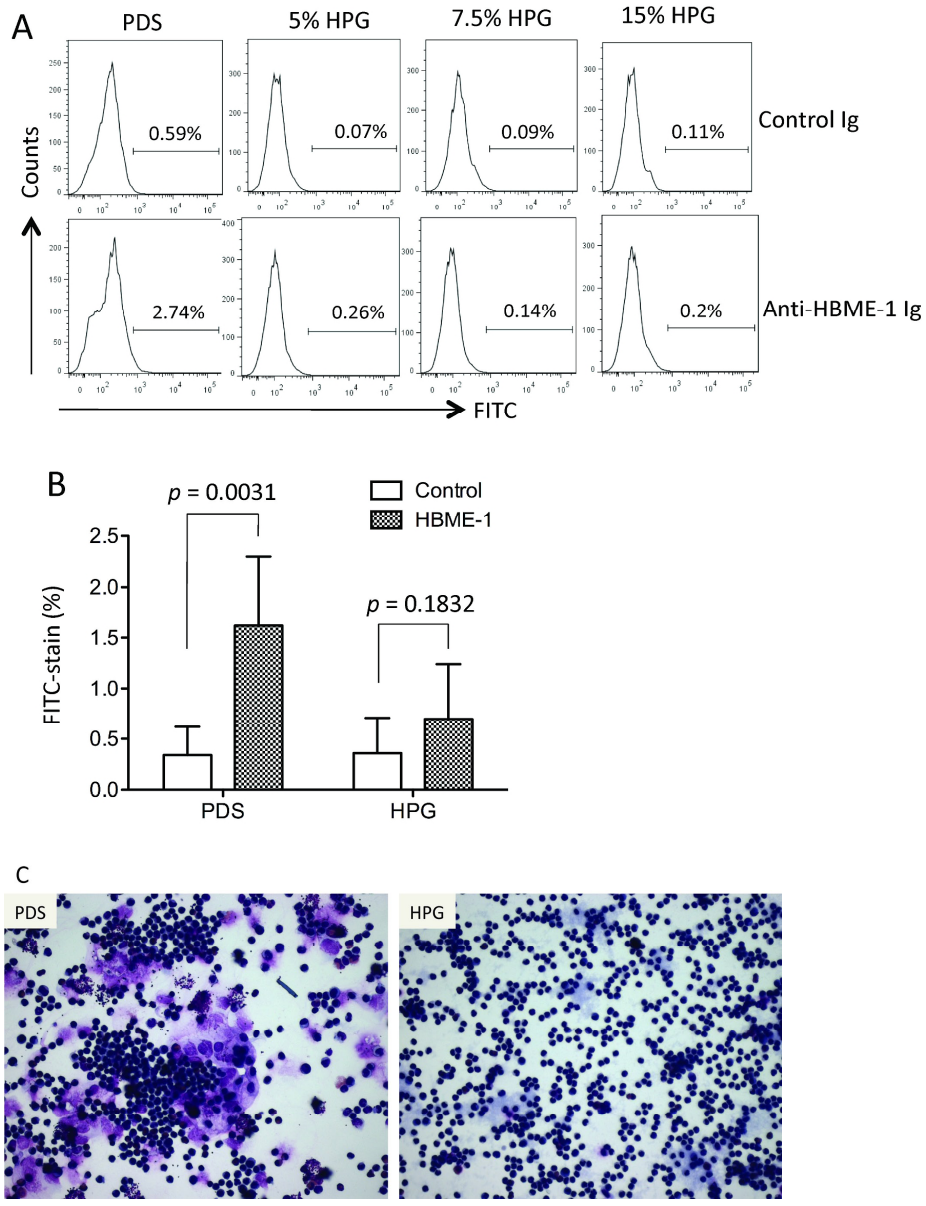

Figure 3.4 HPG solution causes less mesothelial cell injury
Peritoneal membrane injury was determined by the presence of detached HPMCs in the recovered effluent. HPMCs were identified using flow cytometric analysis or MGG stain. (A) Cells were stained with either rabbit anti-mouse IgA polyclonal (control) Ig-FITC or rabbit anti-HBME-1 polyclonal Ig-FITC. Data were presented as a typical percentage of FITC stained cells in FACS histograms of each group. (B) Data were presented as mean ± SD of FITC-stained cells in PDS and pooled HPG solutions (C) A typical microscopic view of MGG-stained smears of cells in PDS versus HPG. Leukocytes: dark blue; HPMCs: blue-violet. Reproduced with permission from Mendelson et al (112).

3.7 Discussion

HPG is a compact dendritic, narrow dispersed macromolecule with low intrinsic viscosity that can be used as a primary osmotic agent in PD solution, indicated by the fact that increasing the concentration of HPG molecule in an electrolyte solution results in an increase in its osmolality (**Table 3.1**). Indeed, significant fluid removal in rats is seen in the dialysis with 7.5% or 15% 3 kDa HPG solution (**Figure 3.1**) for which osmolality is 324 or 424 mOsm/kg respectively (**Table 3.1**); this is higher than the normal range of plasma osmolality in Sprague-Dawley rats (295 ± 3 mOsm/kg) (113). 5% HPG solution (294 mOsm/kg) is considered iso-osmotic to rodent plasma and appears to balance plasma colloid osmotic pressure, resulting in even fluid balance (**Table 3.1, Figure 3.1**).

Small solute transport of all sizes of HPG solutions, measured as D/P urea ratio, was similar to PDS. This supports the biocompatibility of HPG given that increased solute transport may have suggested increased vascular permeability that occurs in acute peritoneal inflammation (114, 115). Moreover, urea clearance values for HPG appear to follow the ultrafiltration profiles observed in our experiments. Nevertheless, in this rodent model of PD, urea clearance of 7.5% 3 kDa HPG solution was significantly higher than PDS despite both solutions having similar ultrafiltration and D/P ratios. This suggests that the exact relationship between solute transport, ultrafiltration, and waste clearance with HPG solutions has yet to be fully characterized.

In our *in vivo* rodent model of PD, HPG solutions induce less peritoneal membrane injury and less leukocyte infiltration (**Figure 3.2, 3.3, 3.4**) compared to conventional glucose-based PD solution. Increased submesothelial thickness after single exposure to PD may correlate better with tissue edema rather than inflammation and should be interpreted

cautiously (116). Hyperosmotic glucose-based PD induces cellular injury to all types of peritoneal cells, including polymorphonuclear cells, phagocytes (*i.e.* macrophage) and HPMCs (66, 108, 109, 117-120). There are some potential explanations for these findings: First, cells may have better survival in the neutral, physiological pH of HPG solutions versus the acidic pH of ~5.2 in PDS. As listed in **Table 3.1**, dissolving HPG in the same electrolyte solution as PDS maintains a physiologic pH, which has been shown to confer survival benefit for HPMCs (69, 121). The ability to easily deliver HPG PD therapy at a physiologic pH should be considered an advantage over conventional glucose-based solutions. Second, since both hypertonic glucose (122, 123) and GDP's (67, 124, 125) can both directly damage the peritoneal membrane and contribute independently to cellular death, the lack of either of these substances in HPG solutions may account for improved biocompatibility.

4. Further Optimization of Size, Concentration, and Electrolyte Profile of HPG Solution.

4.1 Introduction

Although there is some theoretical rationale for selecting the range 1-3 kDa HPG as our osmotic agent (39), the precise selection of 3 kDa as our initial PD osmol was arbitrary. And while one time point is informative, we felt that multiple samplings over a longer period of time would serve to further characterize the fluid kinetic properties of HPG as an osmotic agent. Moreover, the considerable disparity of pH between Dianeal™ and our HPG fluid (**Table 3.1**) leaves unanswered questions regarding the contribution of pH to the biocompatibility of HPG solution. We therefore undertook a second round of acute PD experiments in an effort to refine our optimal candidate PD fluid for long-term PD experiments.

We tested three different sizes of HPG - 0.5 kDa, 1 kDa, and 3 kDa at time points 0.5, 2, 4, and 8 hours. The pH, concentrations, and measured osmolalities can be found in **Table 4.1**. The control solution in these experiments was Physioneal™ solution (2.27% PD4 CAPD solution, Baxter Healthcare Co). This twin-bag glucose-based PD fluid utilizes a lactate/bicarbonate buffer system that maintains the PD fluid at a pH of ~7.4, thus serving as a tighter control for our HPG fluid. The concentrations of various HPG solutions were titrated to approximate the osmolality of PYS 2.27% (~400 mOsm/kg). Biocompatibility testing (flow cytometry) was performed on the 4-hour time point in order to be congruent with the previous round of experiments.

PD Solution	Osmolality (mOsm/kg)	pH
4.8% 0.5 kDa HPG	402	7.4
6% 1 kDa HPG	402	7.6
14% 3 kDa HPG	394	7.4
2.27% glucose, (Physioneal™, Baxter)	395*	7.4

Table 4.1 Biochemical profiles of HPG solutions and Physioneal
Osmolality for HPG solutions was measured in the Vancouver Coastal Health Regional Laboratory and pH was calculated using a laboratory pH meter in a period of 10 min. Values for Physioneal™ were taken from www.medsafe.govt.nz/profs/datasheet/p/Physionealsoln.pdf.
* indicates calculated osmolarity (mOsm/L) instead of measured osmolality (mOsm/kg).

4.2 Ultrafiltration Profile

Figure 4.1 describes the UF profiles for PYS and HPG solutions. All HPG sizes, and every time point induced superior UF when compared to glucose-based PD solution (0.5 kDa vs. PYS, $P = 0.0071$; 1 kDa vs. PYS, $P = 0.0076$; 3 kDa vs. PYS, $P < 0.0001$). The time point of maximal UF for all solutions was 4 hours (PYS: 37.5 ± 1.44 mL; 0.5 kDa: 41.17 ± 3.37 mL; 1 kDa: 41.0 ± 1.0; 3 kDa: 46.67 ± 2.21 mL). All HPG solutions achieved sustained UF up to 8 hours with recovered volumes of 39.67 ± 2.73 mL by 4.8% 0.5 kDa HPG, 38.38 ± 3.16 mL by 6% 1 kDa HPG, and 43.5 ± 3.01 mL by 14% 3 kDa. These values are significantly higher than the initial 30 mL instilled. PYS did not maintain UF at 8 hour and demonstrated a recovered PD volume of 31 ± 2 mL that was similar to the initial instilled volume. These data suggest that HPG (0.5 – 3 kDa) solutions removed more fluid compared to Physioneal at all time points, and more importantly, the ultrafiltration efficacy of HPG solutions was sustained in a prolonged dwell period, while the conventional PYS lost its efficacy.

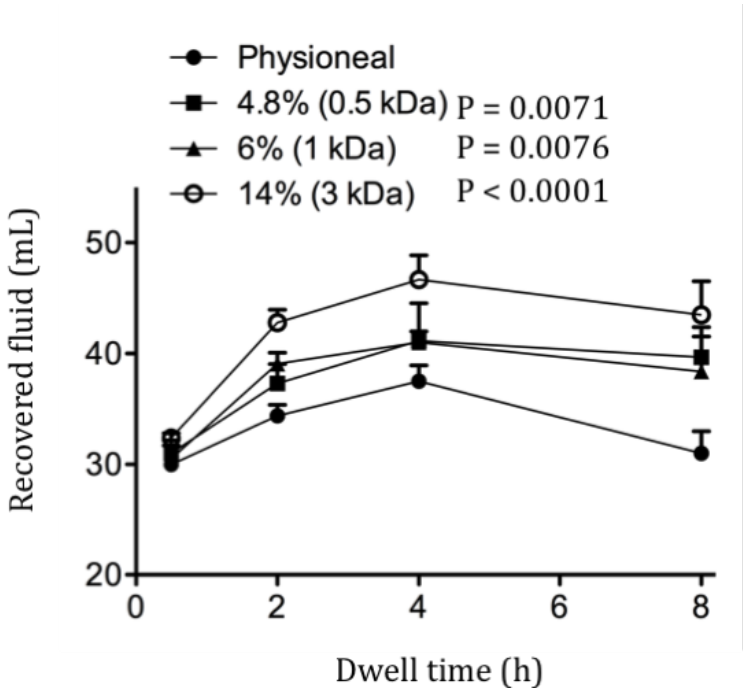

Figure 4.1 Ultrafiltration profiles for HPG solutions and Physioneal
Volume of peritoneal fluid is recovered 0.5, 2, 4, and 8 hours after 30 mL intraperitoneal injection. P-values given for two-way ANOVA of HPG solution vs. PYS. Total 3-5 rats per group.

4.3 Urea Removal

Figures 4.2, 4.3, 4.4 display urea concentration, total urea, urea clearance, and D/P urea ratio achieved by different sizes of HPG solutions and PYS at various time points up to 4h during a PD dwell. 3 kDa HPG was significantly superior to PYS for all parameters measured, with particular efficacy noted at 4 and 8 hours (urea concentration vs. PYS, $P = 0.0038$; total urea vs. PYS, $P < 0.0001$; urea clearance vs. PYS, $P < 0.0001$). By contrast, 1 kDa HPG was superior to PYS only for total urea ($P = 0.0204$) and urea clearance ($P = 0.0097$). 0.5 kDa HPG demonstrated no significant difference in urea removal for any parameters measured ($P > 0.05$). Furthermore, D/P urea values for all groups were not statistically different. Taken together, these data suggest that urea

removal in dialysate is modified significantly by the size of HPG with larger HPG molecules offering superior waste removal than glucose-based solution.

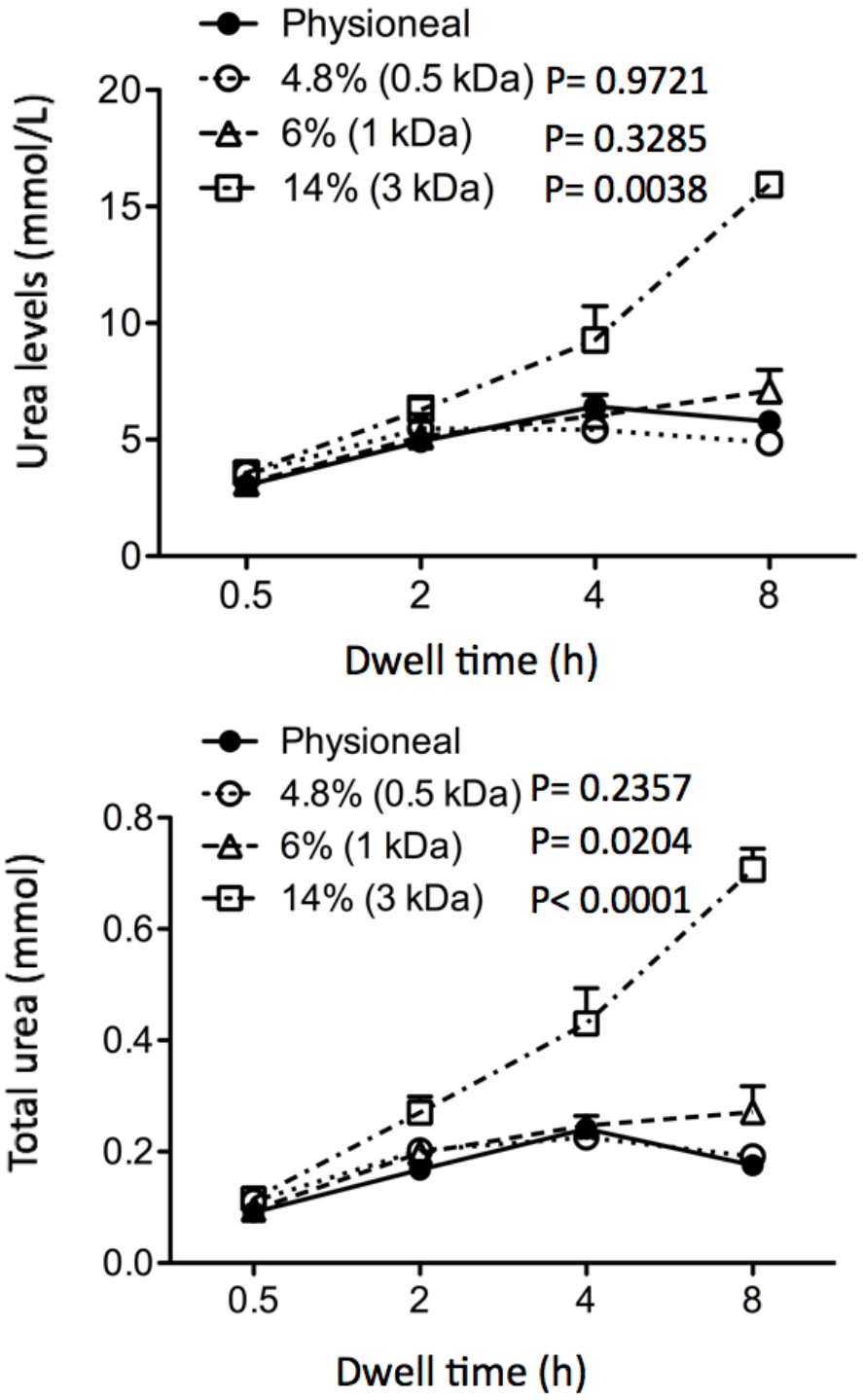

Figure 4.2 Urea concentration (above) and total urea (below) in dialysate for HPG solutions and Physioneal

Urea concentrations (mmol/L) and total urea (mmol) for 0.5 kDa, 1 kDa, and 3 kDa HPG as well as PYS solutions were measured at Vancouver Coastal Health Regional Laboratory for all time points up to 4 hours. P-values given for two-way ANOVA of HPG solution vs. PYS. Total 3-5 rats per group.

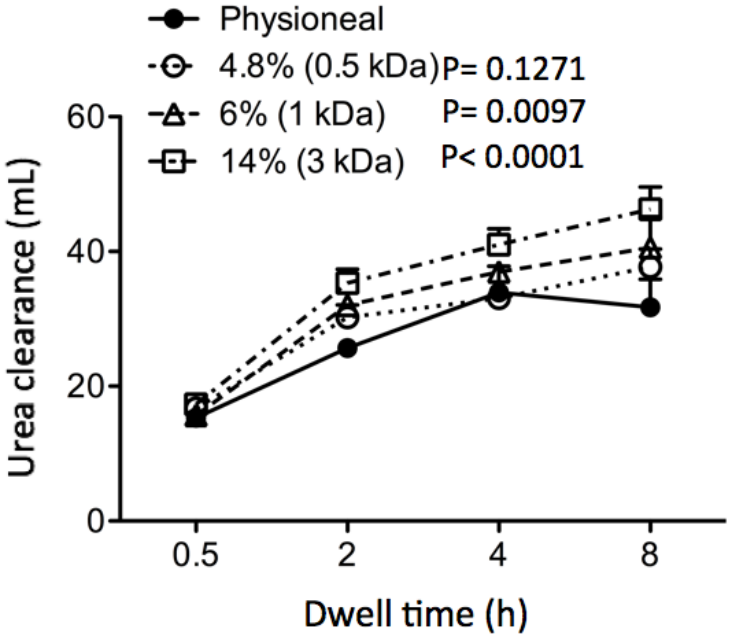

Figure 4.3 Urea clearance for HPG solutions and Physioneal
Urea values in both dialysate and plasma were measured at Vancouver Coastal Health Regional Laboratory. Urea clearance is calculated as $(D/P)_{urea}$ x $V_{dialysate}$ for all time points. P-values given for two-way ANOVA of HPG solution vs. PYS.

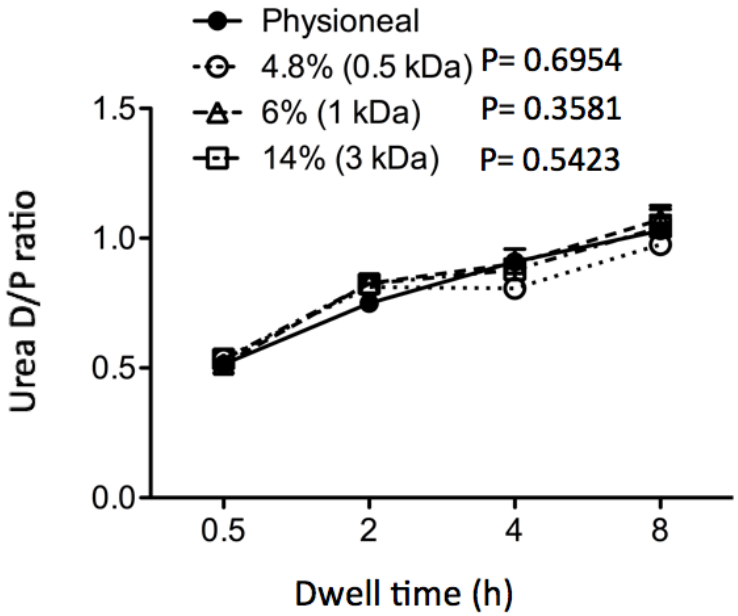

Figure 4.4 Dialysate-to-Plasma (D/P) ratios of urea for HPG solutions and Physioneal
Urea values in both dialysate and plasma were measured at Vancouver Coastal Health Regional Laboratory. No statistical difference was detected between groups ($P > 0.05$ two-way ANOVA).

4.4 Sodium Removal

Figure 4.5 displays the total sodium removal for each PD group at various time points up to 8 hours. All HPG solutions demonstrate superior sodium removal compared to PYS with increasing efficiency in sodium removal with larger sizes of HPG ($P < 0.0001$ for all HPG groups vs PYS).

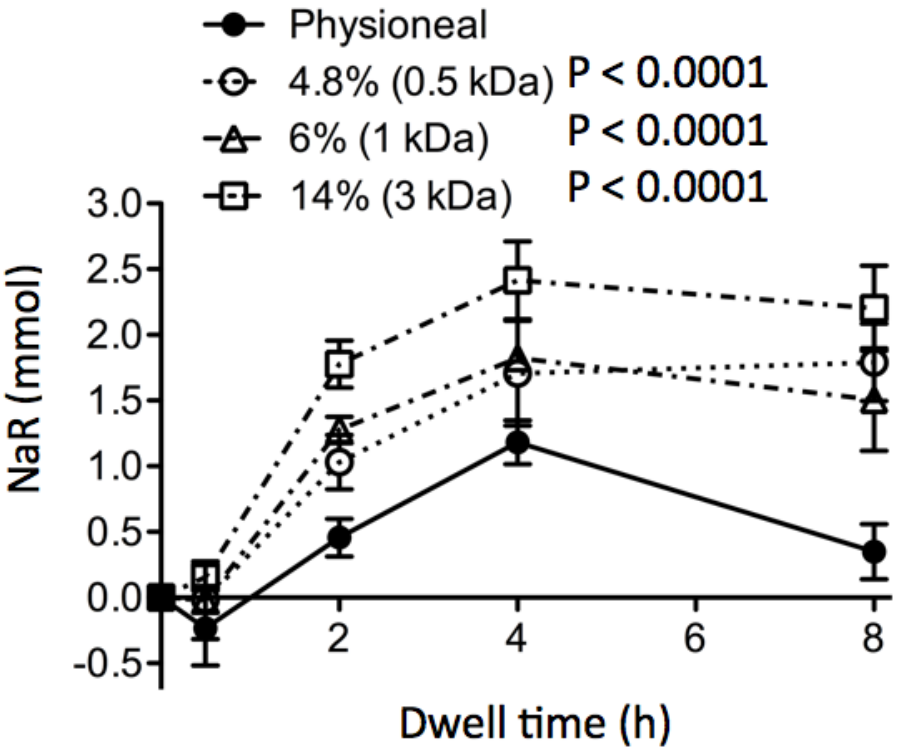

Figure 4.5 Sodium removal for HPG solutions and Physioneal
Sodium values in dialysate were measured in Vancouver Coastal Health Regional Laboratory. Total sodium removal at any time point *t* by PD solution was calculated as $Na_t - Na_{initial}$ whereby $Na = Volume_{dialysate} \times [Na_{dialysate}]$. Initial dialysate volumes were set as 30 mL and sodium values for initial dialysate were measured directly as 138 mmol/L for PYS, 132 mmol/L for 0.5 HPG, and 130 mmol/L for 1 kDa and 3 kDa HPG. P-values given for two-way ANOVA of HPG solution vs. PYS. NaR = Sodium Removal.

4.5 Free Water Transport

Table 4.2 describes the %FWT calculated according to the La Milia method. Hypertonic glucose-based PD solution achieved 37.77 ± 13.19 %FWT, whereas all HPG solutions achieved very little %FWT (-0.752 ± 1.252, -5.621 ± 4.778, -13.62 ± 14.47; for 3 kDa, 1 kDa, and 0.5 kDa respectively). The FWT values for HPG solutions cross the zero value, likely on the basis of elevated serum sodium levels in the rodents for this time point. These differences were statistically significant when compared to PYS ($P < 0.05$) and demonstrate significant aquaporin-mediated water transport for PYS.

	NaR (mmol)	**UFSP (mL)**	**FWT (%)**
Physioneal	0.463 ± 0.141	3.285 ± 1.002	37.77 ± 13.19
4.8% (0.5 kDa)	1.031 ± 0.206	7.499 ± 1.48 (p = 0.0462)	-13.62 ± 14.47 (p = 0.0491)
6% (1 kDa)	1.277 ± 0.101	9.102 ± 0.696 (p = 0.0009)	-5.621 ± 4.778 (p = 0.0185)
14% (3 kDa)	1.778 ± 0.178	12.94 ± 1.313 (p = 0.0004)	-0.752 ± 1.252 (p = 0.0396)

Table 4.2 Free water transport (FWT) calculations for HPG solutions and Physioneal according to the La Milia method
NaR: Sodium removal; UFSP: ultrafiltration through small pores; FWT: free water transport via the aquaporins. P-value of two-tailed t-test indicated the difference between Physioneal and HPG solution (n = 5 for all groups).

Similarly, as shown in **Figure 4.6**, sodium sieving was observed uniformly for all groups at time 0.5 hour with percent drop in sodium concentration -5.625% ± 0.88 for PYS, -4.57% ± 2.00 for 0.5 kDa HPG, -2.15% ± 0.71 for 1 kDa HPG, and -3.65% ± 1.15 for 3 kDa HPG. However, only PYS sustained this sodium sieving at 2 hours (-4.37% ± 1.27) with all HPG solutions recovering their sodium concentrations back to baseline (P =

0.0498 vs. 0.5 kDa; P < 0.0001 vs. 1 kDa; P < 0.0001 vs. 3 kDa). Taken together, these two sets of data support the concept of aquaporin-induced UF for crystalloid PYS (i.e. glucose-based PD fluid) and suggest that HPG-induced UF occurs primarily through small pores, likely on the basis of colloid osmosis.

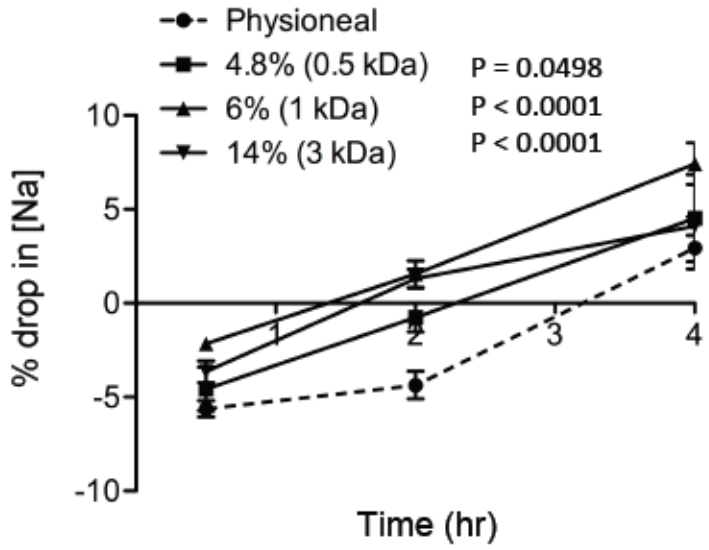

Figure 4.6 Sodium Sieving of HPG solutions and Physioneal
Percentage drop in initial dialysate sodium concentration at time points up to 4 hours. P-values given as two-way ANOVA of HPG vs. PYS.

4.6 Histological Examination

As shown in **Figure 4.7**, peritoneal submesothelial expansion is significantly reduced for 0.5 kDa HPG and 1 kDa HPG sizes when compared to PYS, indicated by the submesothelial thickness 101.8 ± 20.09 µm in PYS group compared to 53.12 ± 5.75 µm in 0.5 kDa HPG (P = 0.0496), 45.35 ± 5.2 µm in 6% 1 kDa HPG (P = 0.0351), or 58.77 ± 7.44 µm in 14% 3 kDa HPG solution (P = 0.0647). Moreover, neutrophil infiltration was observed less frequently on histological examination.

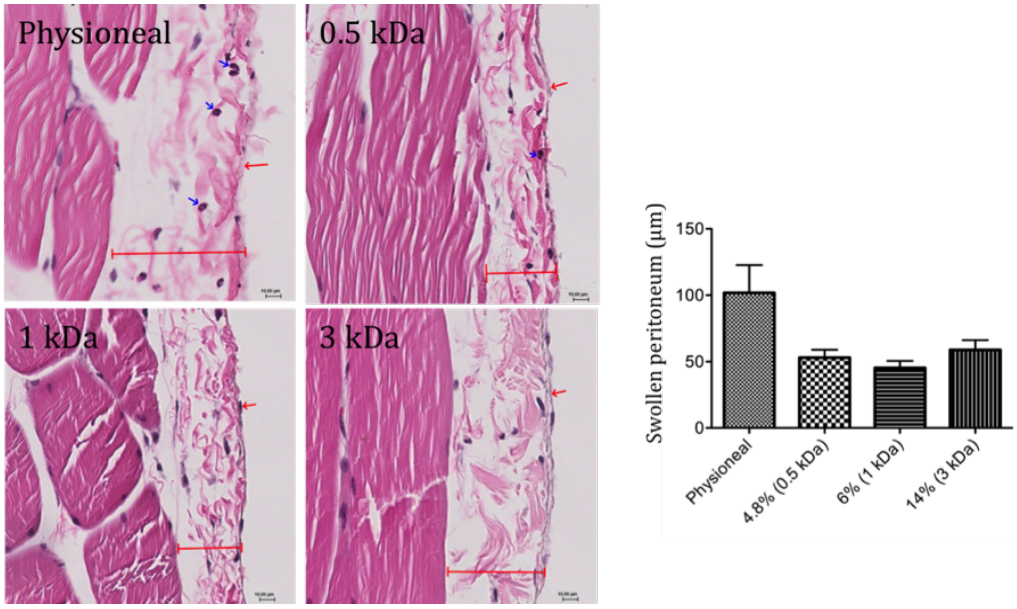

Figure 4.7 Histological examination of the peritoneal membrane after 4 hours of exposure to varying sizes of HPG and PD solutions
The right images are representative of peritoneal membrane in each group. The left figure presents the mean ± SEM of submesothelial thickness in each group after 4 h of dwell (n = 3-4). P-values for two-way t-test versus PYS: 0.5 kDa, P = 0.0496; 1 kDa, P = 0.0351; 3 kDa, P = 0.0647. Blue arrow: neutrophil; red arrow: peritoneal mesothelial cell.

4.7 Neutrophil Response

Figure 4.8 demonstrates the percent neutrophil populations in recovered dialysate after 4 hours of PD exposure as measured with flow cytometry. All HPG sizes demonstrate proportionally less neutrophils (0.5 kDa HPG: 7.31 ± 4.14%; 1 kDa HPG: 3.22 ± 1.7%; 3 kDa HPG: 4.96 ± 1.59%) when compared to PYS (10.4 ± 2.24%) ($P = 0.0036$ vs. 0.5 kDa; $P < 0.0001$ vs. 1 kDa; $P < 0.0001$ vs. 3 kDa).

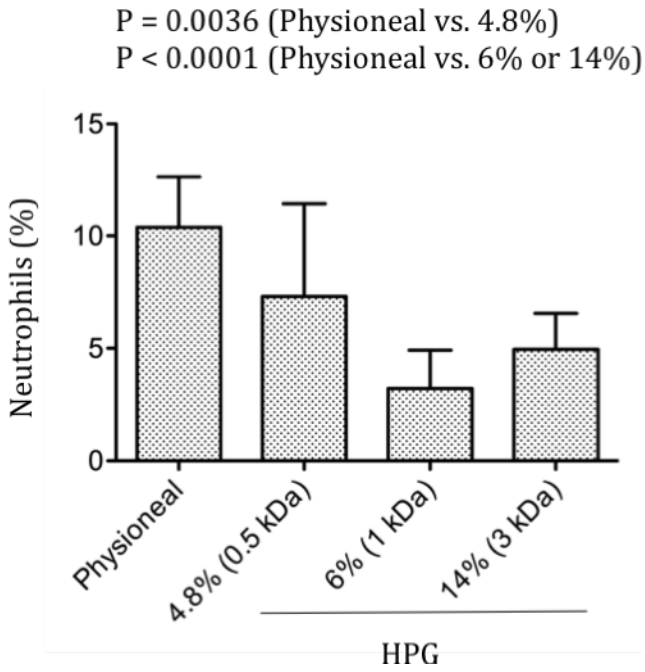

Figure 4.8 All sizes of HPG solution induce less neutrophil infiltration
The presence of neutrophils in the recovered fluids was used as a biomarker for peritoneal inflammation and was quantitatively determined using flow cytometry. Data were presented as mean ± SD of neutrophils in for HPG solutions versus PYS.

4.8 Mesothelial Cell Detachment

In the second round of experiments, mesothelial cell detachment in recovered dialysate was significant for both PYS (P = 0.0041) and 3 kDa HPG (P = 0.0304) but was not elevated for 0.5 kDa and 1 kDa HPG solutions (**Figure 4.9**). Also, fewer total HBME-1 positive cells were seen in all HPG groups including 14% 3 kDa HPG solution as compared to PYS group. These *in vivo* data support the biocompatibility of HPG over glucose-based solutions – irrespective of pH effect. These data suggest that smaller HPG sizes may be better tolerated by the peritoneal membrane.

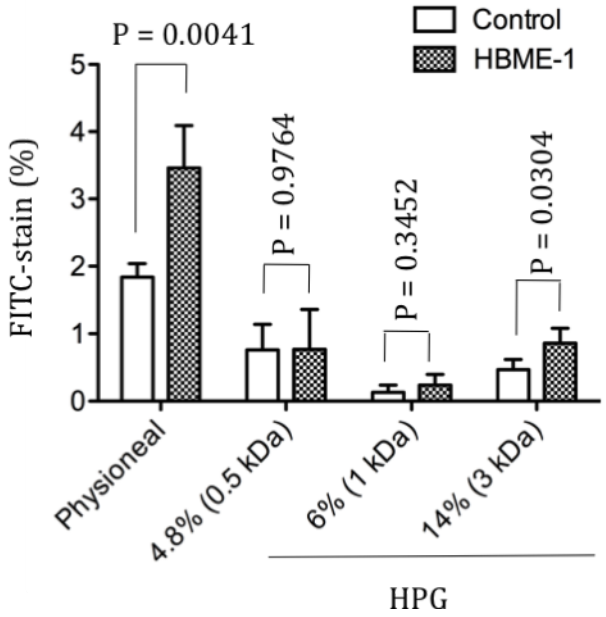

Figure 4.9 Reduced mesothelial cell detachment by 0.5 kDa and 1 kDa HPG solution
Peritoneal membrane injury was determined by the presence of detached HPMCs in the recovered effluent after 4 hours of exposure. HPMCs were identified using flow cytometric analysis. Cells were stained with either rabbit anti-mouse IgA polyclonal (control) Ig-FITC or rabbit anti-HBME-1 polyclonal Ig-FITC. Data were presented as mean ± SD of FITC-stained cells for all sizes of HPG solution and PYS.

4.9 Discussion

This second round of experiments further characterizes the UF profiles and solute removal capacity of HPG-based PD solutions of varying molecular weights. In contrast to glucose-based PD, HPG solutions sustain their UF for up to 8 hours. These results are similar to observations that have been noted with icodextrin (37, 126, 127). We hypothesize that this phenomenon is a direct result of the increased size and reflection coefficient of HPG compared to glucose. HPG is less easily absorbed across the peritoneal membrane during a PD dwell. Thus, HPG-based PD solutions are able to sustain osmotic pressure for an extended period of time; they behave as a colloid. In contrast to icodextrin, which demonstrates a slow linear UF profile and UF peak past 8 hours (37) (**Figure 4.10**), our data show that HPG quickly reaches maximal UF capacity

at 4 hours (**Figure 4.1**). This advantage over icodextrin may allow for HPG polymers to be used multiple times daily for short dwells.

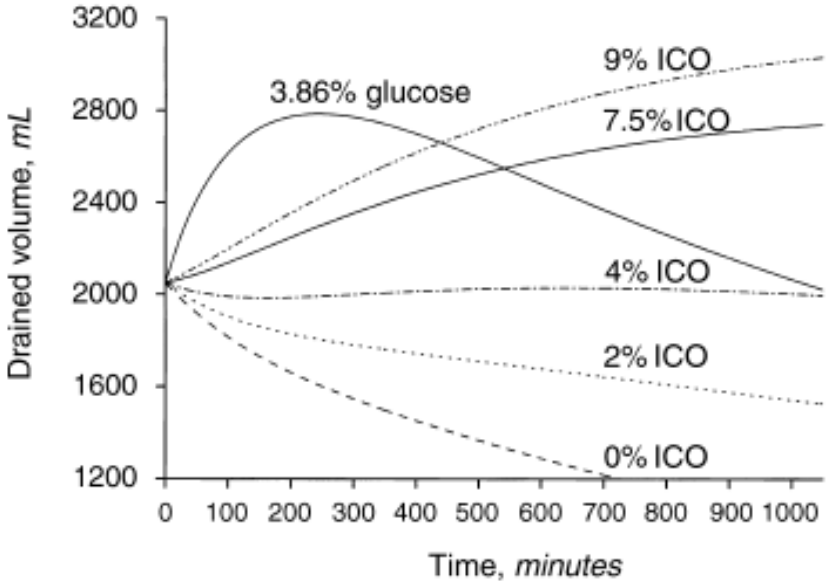

Figure 4.10 Computer-simulated ultrafiltration profiles for hypertonic glucose and icodextrin PD solutions. Reproduced with permission from Rippe et al(37).

The colloidal properties of HPG – as opposed to crystalloid osmosis induced by hypertonic glucose – are further supported by the FWT data. Using two measurements of FWT, we have demonstrated that HPG solutions induce markedly less aquaporin transport than PYS despite superior UF capacity. Thus, it can be concluded that most of the UF is occurring through small pores in a manner similar to icodextrin (40).

Urea removal by different sizes of HPG appears to be highly variable, with 3 kDa HPG offering significant advantage over 0.5 and 1 kDa HPG, as well as glucose-based PD solution. Conversely, PYS and 0.5 kDa HPG had similar waste removal properties despite superior UF capacity of 0.5 kDa HPG. These waste removal properties were encountered despite similar D/P urea ratios for all groups, which indicate that variations in urea clearance did not occur as a result of increasing membrane permeability. The

ability of 3 kDa HPG to remove urea so efficiently may be related to three explanations: First, 3 kDa HPG induced the greatest UF, so it would be expected that solvent drag (i.e., convection) would remove the most urea for this group (36). Second, although all HPG behave as colloids, 3 kDa, as the largest molecule, would most favor the small-pore mechanism of water transport; even in comparison to 0.5 kDa HPG, 3 kDa HPG would tend to drag more urea. Third, it is possible that larger sizes of HPG may actively adsorb urea as a consequence of numerous hydroxyl groups that associate with the urea molecules. This theory is speculative and requires more investigation, but is supported by the linear waste removal properties of 3 kDa HPG, which can be viewed as an outlier compared to the other PD solutions tested in these experiments.

Colloids such as icodextrin has shown in computer modeling to remove more sodium than hypertonic glucose (128), and indeed, clinical studies have demonstrated that substituting icodextrin for glucose in PD prescription has led to improved 24-hr sodium removal PD patients (129). Similarly, all our HPG solutions achieve more effective sodium removal than PYS, which can be explained by a combination of factors: first, as net UF is superior for HPG solutions, it can be expected that solvent drag will also induce superior sodium removal. Second, the starting sodium concentration for HPG solutions was lower than PYS, which facilitates sodium diffusion by creating a larger concentration gradient between dialysate and bloodstream. Last, HPG solutions induce less sodium sieving and less aquaporin-mediated FWT, so more UF is coupled with sodium removal for HPG as compared to PYS. Total UF, plasma-to-dialysate sodium gradient, and absence of sodium sieving have all been shown clinically to correlate with enhanced

sodium removal (130). Our present study corroborates these findings and supports the use of HPG as a superior osmotic agent for sodium removal.

There is a distinct gap between the measured osmolalities of HPG solutions and their theoretical values based on chemical calculation. Despite all measured osmolalities having values ~400 mOsm/kg, the osmolarities of HPG solution are calculated as %wt / mW + 270 (electrolyte osmols) and yield values of 362 mOsm/L, 330 mOsm/L, and 317 mOsm/L for 4.8% 0.5 kDa, 6% 1 kDa, and 14% 3 kDa HPG respectively. Note that the "osmol gap" appears to widen with larger sizes of HPG polymer.

However, freezing point depression was likely a suboptimal choice for measured osmolality for HPG solutions. It has been previously demonstrated with varying sizes of polyethylene glycol (PEG) polymer that the relationship between concentration and osmolality does not follow a linear approximation (131). The relationship is better fitted to an exponential model with larger PEG polymers inducing a greater exponential rise in osmotic pressure for a given molar concentration. Furthermore, the measurement of osmolality for PEG polymers appears to be highly temperature-sensitive (132, 133).

Our FWT data appear to support this theory that HPG solutions are not achieving hypertonic concentrations equal to PYS (~400 mOsm/kg). Despite their large size, it would still be anticipated that markedly hypertonic solution would induce some aquaporin-mediated FWT and sodium sieving. As has been suggested by other authors concerning our published data (80), this osmol gap could theoretically be explained if HPG becomes polydispersed (i.e. disintegrated) in aqueous solution and therefore numerous smaller fractions of HPG contribute to measured osmolality. For example, icodextrin exists in the dialysis solution as a polydispersed molecule of varying molecular

weights (PDI = 2.6) and loses osmotic efficiency compared to the same concentration of monodispersed polymer (37, 134). However, our rigorous chemical analysis clearly indicates this is not the case; HPG is narrowly dispersed with a Mw/Mn (polydispersity index) = 1.1, as measured in chapter 3 and again in chapter 4. Thus, we conclude that in future experiments, vapor pressure osmometry may be a more accurate assessment of the osmolality of HPG solutions and may correlate better with the theoretically calculated osmolarities based on wt% and electrolyte composition (131).

Our HPG PD solutions continue to demonstrate superior biocompatibility over a range of sizes when compared to glucose-based solutions as evidenced by reduced neutrophil infiltration and mesothelial cell detachment observed in the second round of experiments. These findings are independent of pH, given that the control in this second round of experiments was a glucose-based solution with physiologic pH similar to HPG. This concept is supported by rodent data in other studies that concluded that pH of PD solution did not influence the long-term biocompatibility profile (135). It appears that 3 kDa HPG does not offer significant biocompatibility advantage over PYS in the second round of experiments except for reduced neutrophils in recovered dialysate; this is contrasted with the encouraging data presented in chapter 3 for varying concentrations of 3 kDa HPG. The differing results can potentially be explained by the change in control solution from PDS to PYS. Although long-term biocompatibility may not be influenced by pH, perhaps our short-term experiments are influenced by these factors. Moreover, in the second round of experiments, submesothelial thickness was determined quantitatively over multiple histological samples using Slidepath Software (see section 2.7) and therefore may have provided a more accurate assessment of peritoneal membrane response to PD

fluid exposure. Alternatively, in the second round of experiments, we selected a very high concentration of 3 kDa HPG (14 wt%) – much higher than the pooled average wt% evaluated originally. Therefore, 14% 3 kDa HPG may be approaching the upper tolerated limit in this rodent model. It should be noted that in the original experiments, 15% 3 kDa HPG was only slightly more efficacious than 7.5% 3 kDa HPG with respect to ultrafiltration and urea removal. Thus, it is unlikely that doses close to 14% 3 kDa HPG would be used in future experiments and/or clinical situations. Ultimately, it can be concluded that the superior biocompatibility of HPG over glucose in PD solution is an intrinsic property of the polymer/solution and related to reduced glucose exposure to the peritoneal membrane, which has repeatedly been demonstrated to negatively impact the biocompatibility of PD solutions (122, 123, 136).

5. Conclusion

5.1 Summary of Results

We have demonstrated in a series of *in vivo* experiments that a novel HPG-based PD solution can achieve successful ultrafiltration, solute, and waste removal while displaying reduced peritoneal injury and inflammation compared to conventional glucose-based PD solution and glucose-based PD solutions with physiological pH. HPG PD solutions achieve sustained ultrafiltration for up to 8h, which is a distinct advantage over glucose solutions that lose their UF capacity over extended periods of time. The colloidal nature of HPG offers superior sodium removal and urea clearance, which may benefit in long-term volume control and dialysis adequacy for PD patients. Taken together, these preliminary studies suggest that HPG may be a promising alternative to glucose in the development of next-generation PD solutions for patients with end-stage renal disease.

5.2 Experimental Limitations

As an initial proof of concept for our novel HPG solution, the current animal model in these experiments simulates only one dwell of PD. As such, these experiments are limited to only basic markers of short-term biocompatibility. Ultimately, superior biocompatibility of HPG solution can only be established if the reduction of inflammation and peritoneal injury observed in these experiments translates into reduced fibrosis, epithelial-to-mesenchymal transition, and decreased angiogenesis over many weeks of exposure (63, 65, 73, 135, 137, 138). In long-term animal experiments, the presence of uremia also becomes more pertinent – particularly when studying the pharmacokinetic properties of HPG. Furthermore, given the high level of amylase within the rodent peritoneum, we were unable to use icodextrin as a control for our experiments

because it is very quickly degraded into smaller fractions (139). As authors have stated, it is also not currently known whether HPG is broken down within the peritoneum (80), nor is it clear how HPG will affect the metabolism of ESRD patients.

5.3 Future Research

Our ongoing research into HPG PD solutions is focused on chronic animal models and daily peritoneal exposure to HPG solutions to assess the long-term biocompatibility of our HPG PD solution. We are also exploring different animals models of renal insufficiency and diabetes in order to assess the organ accumulation, systemic metabolic effects, and hepatic excretion of HPG in a preclinical setting that more closely approximates ESRD. Radiolabeling of HPG will help us determine how the size of the polymer influences its absorption across the peritoneal membrane.

We are developing a series of experiments that will allow us to bridge the gap between laboratory experimentation and clinical testing. Our hopes are that a biocompatible, glucose-sparing HPG solution will soon be available to improve the care that we deliver to PD patients.

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Appendix

Calculation of Free Water Transport according to the La Milia Method (42)

$$FWT\ (mL) = Total\ UF\ (mL) - UFSP\ (mL)$$

$$UFSP\ (mL) = [NaR\ (mmol) \times 1000] / Na_{Plasma}\ (mmol/L)$$

$$NaR = [V_{DialysateOut}\ (L) \times Na_{DialysateOut}\ (mmol/L)] - [V_{DialysateIn}\ (L) \times Na_{DialysateIn}\ (mmol/L)]$$

$$\%FWT = [FWT / Total\ UF] \times 100$$

FWT = Free Water Transport; UF = Ultrafiltration; UFSP = Ultrafiltration through small pore; NaR = Total Sodium Removed; Na = concentration of sodium; V = Peritoneal Dialysate Volume; %FWT = Percent Free Water Transport