CHAPTER III

MATERIALS AND METHODS

3.1 TPDF composition and its degradation products

Tapioca derivative was obtained from Ban Pong Company. The molecular characteristics were reviewed. Varies concentrations of tapioca-derived based PDFs (TPDFs) were prepared in our laboratory. The composition of TPDF was similar composition with commercially available PDFs included as follows: NaCl 5.40g/L, $CaCl_2 \cdot 2H_2O$ 0.257g/L, $MgCl_2 \cdot 6H2O$ 0.508g/L, sodium D/L-lactate 4.48g/L, and anhydrous D-glucose 15.0 or 42.5g/L. The solutions were divided into two volumes that were sterilized either by heat (121°C, 0.2 MPa, 20 minutes) or by filtration through 0.2 μm pore-size filter (Corning Incorporated; Corning NY 14831, Germany). Osmolality and pH, before and after sterilization, were measured. A fresh TPDF prepared with 1 month storage in a refrigerator was used. In order to eliminate the well-known inhibitory effects related from low pH; all PDFs were neutralized to pH 7.3 with 0.1mol/L $NaHCO_3$ prior to use in cytoxicity the experiments.

Commercially-available glucose-based peritoneal dialysis fluid (GPDFs) 1.5% and 4.25% dextrose (from Baxter, Health Care, Philippines INC) 100ml solution containing 1.5g or 4.25g dextrose hydrous USP, NaCl 5.38g/L, CaCl2 • 2H2O 0.257g/L, MgCl2•6H2O 0.508g/L, sodium D/L-lactate 4.48g/L, pH 5.2 and glucose polymer corn-based peritoneal dialysis fluids (CPDFs) 7.5% (from Baxter Health Care, Singapore Branch) were used to compare to our developed TPDF.

The effect of preparation and storage on the TPDF stability was evaluated. The glucose degradation products after preparation were simultaneously quantified. Samples after autoclaving were stored in a refrigerator (2-8°C), at room temperature (25-28°C) and at 37°C baseline and for 1, 3, 6, 12, 18 and 24 months. Four major GDPs, Glyoxal (GO) and Methylglyoxal (MGO) and 3-deoxyglucosone (3-DG), 5-Hydroxymethyl-furaldehyde (5-HMF) occurred during the heat sterilization process and

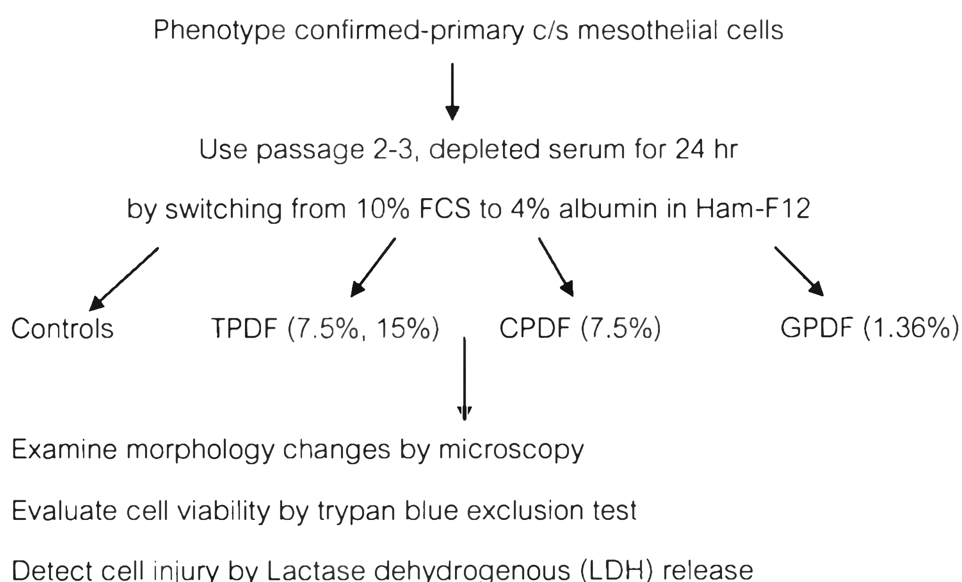
storages were quantified with reference to the methods of Wieslander *et al* and 3-DG according to [212].

3.2 Safety testing

3.2.1 Cytotoxicity Studies

A. Human mesothelial cells testing

Human mesothelial cells culture: the HPMCs were isolated, characterized, and maintained in culture as described in detail elsewhere [126]. Briefly, the primary human peritoneal mesothelial cells (HPMC) were harvested from omentum, collected from peritoneal dissemination elective during abdominal surgery. The cells were propagated in the completed HAM-F12 culture medium, supplemented with L-glutamine (2mmol/L), penicillin (100U/mL), streptomycin (100μg/mL), hydrocortisone (0.4μg/mL), and 10% v/v fetal calf serum (FCS). The cells were grown and maintained in 37°C incubators with a humidified atmosphere of 95% air and 5%CO_2. Injury, cell viability and cell death of mesothelial cells were tested. Because HPMC were characteristically stable from the second and third passage, all experiments were performed using cells in the 2nd or 3rd passage.



HPMC morphology changes study: The 2-3 propagated 90% confluent monolayer HPMCs were used to examine cytotoxicity. $5x10^4$ cells/well was seeded onto round glass microscope slides and grown in 24-well-plates in a 500μl volume. Then, to arrest and synchronize cell growth, the cells were depleted to serum for 24 hours. Albumin was added to all PDFs in 4% final concentration. Mitomicin C (200μg/ml) was used as positive treatment and diluted in Dulbecco's Modified Eagle's Medium (DMEM); a high glucose solution was used as positive PDFs controls. After that, the cells were incubated with either control culture medium or fresh pH-neutralized PDFs for specified time periods 30 minutes, 4, 8 and 24 hours. Afterward, up to 36 hours, he changing of cell's morphology was inspected under microscopy. The photos at specific time points were taken.

LDH released to determine HPMC injury: cell membrane integrity was measured. LDH is a cytoplasmic enzyme that is released into the cytoplasm if having cell lysis. Therefore, LDH assay was used to evaluate cytotoxicity. The basic principle of the LDH assay: (i) LDH oxidizes lactate to private; (ii) Pyruvate reacts with the tetrazolium salt to form formazan; and (iii) the water-soluble formazan dye is detected spectrophotometrically. The assay was performed using a commercial kit according to manufacturer's instruction (CytoTox 96, Promega, and Madison, WI, USA).

Cell integrity was evaluated immediately after being exposed to PDFs for 15-18 hours. The assay was performed following the trypan blue exclusion test and according to manufacturer's instruction. Briefly, 50 microliters of supernatant was transferred to ninety-six well-plates and incubated, in triplicate, with reconstituted substrate mixture, covered with foil and incubated at room temperature for 30 minutes at 37°C. The reaction was then stopped with stop solution. Optical density was measured at 492 nm (OD492) within one hour. LDH release was calculated as a percentage of the gradient obtained from an equivalent number of cells on the same day cell propagations. The dialysis fluids alone did not affect the magnitude of optical density change.

% Total LDH Leakage =

$$\frac{(\text{sample absorbance} - \text{cell free sample blank}) - \text{mean media control absorbance}}{\text{mean TritonX positive control absorbance} - \text{mean media control absorbance}} \times 100$$

Mean, SD and %CV should be calculated for each positive control, negative control and unknown sample.

HPMC death by Flow Cytometry

After the 18 hours PDFs incubation procedure, the cells were harvested with 0.5% trypsin, washed with an ice-cold PBS. 1–2 × 10^6 cells were suspensed in 0.5 mL of phosphate-buffered saline (PBS). The suspension of the cell was added into 5 mL of 1% (w/v) paraformaldehyde in PBS and placed on ice for 15 minutes, then centrifuged the cells for 5 minutes at 300 × g and discarded the supernatant. Cells were washed by 5 mL of PBS with same rate of centrifugation. Then the cells were diluted in 0.5 ml of PBS. Then cells were fixed by adding of 5 mL of ice cold 70% (v/v) ethanol for a minimum of 30 minutes on ice or in a –20°C freezer. In some biological systems, fixed cells were storage at –20°C in 70% (v/v) ethanol for at least 12–18 hours prior to performing the TUNEL assay that yields the best results. Cells can be stored at –20°C for several days before use.

The fixed cells were incubated with the Fluorescein anti-BrdU Antibody Solution in the dark fr 30 minutes at room temperature. Hint: Wrap tubes with aluminum foil. Then, 0.5 ml of the Propidium Iodide/RNase A Solution (amber bottle) was added to the tube containing the 0.1 ml Antibody Staining Solution Note: If the cell density is low, decrease the amount of PI/RNase A solution to 0.3 ml. The cells were incubated in the dark for 15 minutes at room temperature. Apoptotic cells in Propidium Iodide/RNase Solution were analyzed by flow cytometry. Minimum of 50,000 cells were acquisition within 3 hours of staining.and analyzed by flow cytometer (FACS Calibur; Becton Dickinson). Dot plot analysis was used. HAM-F12 served as the negative control. Cells exposed to high glucose PDF was used as positive control. Intra-assay coefficients of variation were less than 5% (n=5).

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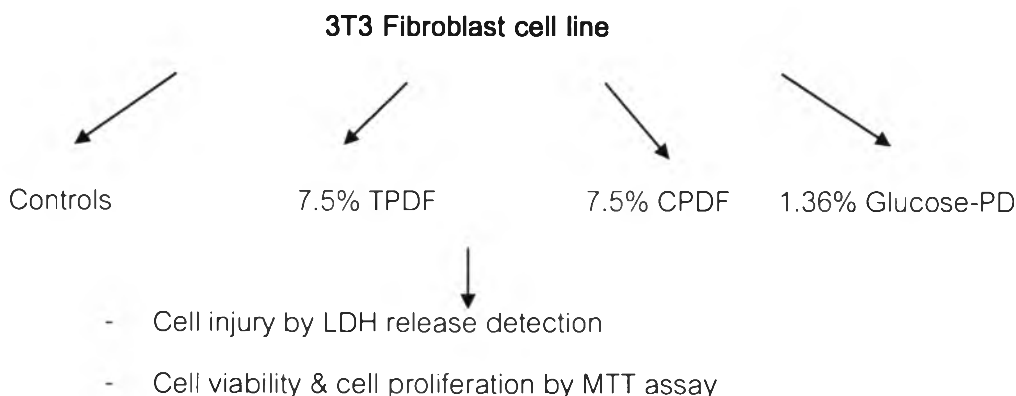
B. Repeat cytotoxicity testing

3T3 fibroblast cell culture: 3T3-Swiss albino is an adherent fibroblast cell line which was disaggregated from an embryo Swiss mouse. This cell line was obtained from ATCC, CCL-92™. It was cultured in DMEM medium supplemented by 10% FCS, L-glutamine (2mM), penicillin (100 U/ml), streptomycin (100 U/ml), and hydrocortisone (0.4 U/ml). The cells were grown and maintained in 37°C incubators with a humidified atmosphere of 95% air and 5%CO_2.

The 3T3 cell line: cell viability and cell proliferation and death



Cell injury by detection of LDH release: Briefly, $1x10^5$ cells/ml was dispensed in 96-well plate. Cells were cultured in completed DMEM medium for 24 hours. Before cells were exposed to PDFs, they were washed once with FCS-free medium. A fresh prepared pH-neutral PDF was mixed with an equal volume of control medium supplemented with 4% albumin to maintain baseline cell viability.

Cells were treated with PDFs for 60 minutes, 50 microliters of supernatant was transferred to ninety-six well-plates and incubated, in triplicate, with reconstituted substrate mixture, covered with foil and incubated at room temperature for 30 minutes at 37°C. The reaction was then stopped with stop solution. Optical density was measured at 492 nm (OD492) within one hour. LDH release was calculated as a percentage of the gradient obtained from an equivalent number of cells on the same day cell propagations.

Cell viability by MTT assay: To determine cell viability the colorimetric MTT metabolic activity assay was used. The MTT was used to assess cell viability. MTT is a

yellow, water-soluble tetrazolium dye that is reduced by live cells to a water-insoluble, purple formazan. The amount of formazan can be determined by solubilizing it in DMSO and measuring its optical density.

A 50μl portion of secondary culture cell solution (containing $1x10^5$ cells/ml) was dispensed in 96-well plate. Cells were cultured in completed DMEM medium that contained 50 U/mL penicillin and 50 mg/mL streptomycin, 10% FCS and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. Before cells were exposed to PDFs, they were washed once with FCS-free medium. A fresh prepared pH-neutral PDF was mixed with an equal volume of control medium supplemented with 4% albumin to maintain baseline cell viability.

The cells were incubated with the prepared PDFs at volume 50μl per well for 30 minutes and 6 hours, then assessed for viability (MTT test). After treatment, the supernatant of each PDF was discarded, and then 150μl of medium and 50μl of MTT (5mg/ml) were placed. The plates were incubated at 5% CO2 at 37°C for another 4 hours. After that, a 150μl portion of the supernatant was carefully aspirated. Dissolution of formazane in 150μl of dimethyl sulfoxide (DMSO) was added. Cell viability was quantified by detecting cleavage of MTT. The amount of formazan can be determined by solubilizing it in DMSO. The absorbance intensity measured by a microplate reader (Bio-RAD 680, USA) at 490 nm with a reference wavelength of 620 nm. All experiments were performed in quadruplicate, and the relative cell viability (%) was expressed as a percentage relative to the untreated control cells.

3T3 cell proliferation by MTT assay: the effect of solutions on cell proliferation was evaluated. Briefly, the 3T3 cell line monolayer was washed with PBS, and 0.25% trypsin solution was used to detach the cell adherent. Then cell viability was counted by trypan blue in a hemocytometer. The cell were plated onto 96-well plate at a density of 2000 cells per well and cultured for 24 hours in DMEM that contained 50 U/mL penicillin and 50 mg/mL streptomycin, 10% FCS.

The cell cultures were then exposed to the PDF being tested mixed with an equal volume of the standard culture medium supplemented with 10% FCS to stimulate

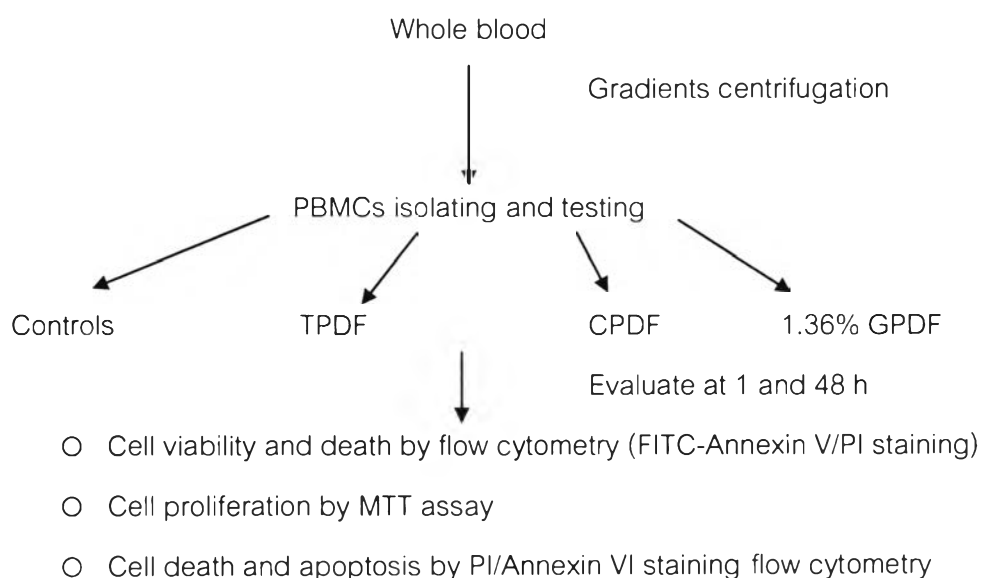
the cell proliferation. A test mixture (200uL) was applied to each well, and plates were incubated for between 48-72 hours. Then the supernatant of treated cells was discarded and washed with FCS-free media, 50 ul of MTT solution was added and incubated for 2 hours in 37°C incubator 95%CO_2. The absorbance was read at 570 nm. The average cell growth as a percentage of growth was determined for each solution.

$$\% \text{ Cell proliferation} = \frac{\text{sample absorbance} - \text{cell free sample blank}}{\text{mean media control absorbance}} \times 100$$

C. Peripheral blood mononuclear cell (PBMCs) testing

PBMC preparation and culture: PBMCs were isolated from the EDTA-blood of healthy volunteers by FCPDFII-Hypaque density gradient centrifugation, as described previously [213]. It was isolated as soon as possible after collection or kept for not over 6 hours at 20-28°C until isolation. In short, 10 ml of whole blood was over-layered gently on 5 ml of FCPDFII-Hypaque solution with density of 1.077 and centrifuged at 690 g, with no break for 20 minutes. The red blood cells were in the bottom of the tube, and the interface PBMCs were collected and washed in 10ml of RPMI by centrifugation at 250g. Then, the cells were washed and incubated with prepared PDFs and grown at 37°C for 6 and 24 hours in a humidified atmosphere of 95% air and 5%CO_2.

PBMC viability, proliferation and apoptosis



- Cell viability and death by flow cytometry (FITC-Annexin V/PI staining)
- Cell proliferation by MTT assay
- Cell death and apoptosis by PI/Annexin VI staining flow cytometry

Cell viability and death by FITC-Annexin V/PI double staining: five million PBMC were incubated in 5mL of dialysis fluid with and without pH-adjusted for 30 minutes at 37°C. The cells were removed by low speed centrifugation (300xg). The reproducibility and stability of the assay was verified. A staining step was performed. After washing the treated cells twice with PBS, 1×10^6 treated cells were suspended in a binding buffer (10mM Hepes/ NaOH, pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$,). Then the cell suspension was incubated with FITC labeled annexin V (final concentration of 2 ug/mL) and PI (final concentration of 1 ug/mL) for 10 minutes in the dark at 4 degree. DNA was binding to perpodium iodine (PI) and FITC-Annexin V. The stained 100,000 cells were measured by FACSCalibur using Cell Quest software. Dot plot analysis was used. M199 served as the negative control. Cells exposed to high glucose PDF was used as positive control. Intra-assay coefficients of variation were less than 5% (n=5).

Cell proliferation by MTT assay: PBMC were washed with PBS, and then cell viability counted in a hemocytometer before seeding into 96-well tissue culture plates at a concentration of 2000 cells/well. The seeded cells were allowed to grow for 24 hours in MEM containing 50U/mL penicillin and 50mg/mL streptomycin, 10% FCS. Test solutions were prepared by mixing PD solution with MEM (without serum) at a 1:1 ratio, and adding FBS to a final concentration of 10% (v/v), with pH adjustment of the test solutions to 7.4. Test solution mixtures (200 mL) were applied to each well, and plates were incubated for approximately 1 and 24 hours.

The treated cells were then supernatant-discarded and washed with FBS-free media, had 50ul of MTT solution added and were incubated for 2 hours in 37°C incubator 95%CO2. The absorbance was read at 570 nm. Average cell growth as a percentage of growth was determined for each solution.

$$\text{\% Cell Viability} = \frac{(\text{Sample absorbance} - \text{cell free sample blank}) \times 100}{\text{Mean media control absorbance}}$$

3.3 Animal Toxicity Testing

Test materials: 0.85% NaCl, 7.5% CPDF, 7.5% TPDF were prepared. The test materials were kept in the dark at ambient temperature when not in use.

Animals and management: animal models provide an important study tool for the biocompatibility of PD Fluids testing. Small mammals are mostly used: rabbits, rats, and mice are economical, easy to obtain and keep [214-216].

For this study, mice, aged 5-7 weeks old, were used. The animals weighed 27-40g, and were housed for acute and chronic toxicity studies. The animals were sourced from the National Laboratory Animal Centre, Mahidol University (NLAC-MU). During the course of the study, the animals were housed, 4-5 animals together, in a suspended polypropylene cage with wood shavings as bedding material at the Laboratory Animal Centre, Faculty of Medicine, Chulalongkorn University.

The environment temperature was 20-25°C and humidity about 46%. A 12h light/dark cycle was in operation. Environmental conditions were monitored daily. The animals were allowed an acclimatization period of 7 days before test commencement. Food and tap water were available and libitum throughout the study. The food and drink used were considered not to contain contaminants in sufficient concentration to have had any influence on the outcome of the study. Each animal was offered approximately 50mg a day.

3.2.2 Acute toxicity in animals

A. Acute toxicity testing in mice (intravenous)

Male and female mice were used for acute 14-day intravenous toxicity tests. Mice were injected with 15% tapioca-based glucose polymer (N=8) at dose 5ml/Kg and, compared with a control group which was injected with 0.85% sodium chloride (N=3).

B. Acute toxicity testing in mice (intraperitoneal)

Male and female mice were used for acute 14-day intraperitoneal toxicity tests. Mice were injected with 15% tapioca-based glucose polymer (N=10) at dose 10ml/, compared with a control group which was injected with 0.85% sodium chloride (N=10).

During the experiment, clinical signs and body weight mortality were observed. Necropsies, organ weight, organ weight per body weight and pathology examination were carried out.

Pathological examination was described. Tissue specimens obtained at the time of sacrifice were used. Tissues were stained and viewed by light microscopy employing ×10 flat field objectives and ×40 flat field objectives, and a drawing apparatus (Olympus, Tokyo, Japan). Each tissue section was measured at ten random locations by two blinded observers using the standardized scoring system (see appendix)

3.4 Effectiveness of this TPDF-induced water transportation

Normal blood-donated aphaeresis plasma was obtained from the Thai Red Cross. The experiments were performed using the same batch of SnakeSkin® Pleated Dialysis Tubing molecular weight cutoff (MWCO) 3.5-kDa and 10-kDa purchased from Thermo Scientific (www.piercenet.com). Dialysis tubing length of 12cm was used in all experiments.

Dialysis solutions: 7.5% corn-based PDF with osmolarity 280 mOsm/L from (Baxter Health Care, Singapore Branch) as a HMW and GPDF at a concentration of 1.5% and 4.25% dextrose with osmolarity 346 and 485 mOsm/L (from Baxter, Health Care, Phillippines INC) as a LMW osmotic agent were used.

3.4.1 Efficacy of TPDF on water osmosis compared to GPDF and CPDF

Tapioca derivative is a glucose polymer hydrolyzed from cassava starch with a heterogenous molecular weight distribution the same as corn derivative glucose polymer. Whether TPDF can induce water osmosis was performed compared to GPDF and CPDF. Water osmosis induced by TPDF, GPDF and CPDF were performed. Cellophane bags having MWCO 3.5 and 10 kDa (mimic the simple peritoneal pore sizes) containing of 5 mL of individual triplicate PDF were placed in 30 mL either distilled water and plasma.

Each PDF (TPDF, GPDF and CPDF) was contained in cellophane bags with 3.5kDa and 10kDa MWCOs and the bags were placed in 30ml water containers and

plasma containers (as shown at Fig. 3.1) with a slow movement (70rpm). The cellophane bags were weighed at the same dwell time. At five minutes, each bag's mass was weighed as baseline. Then, the mass increase induced by dialysis fluids with different dwell times (30, 60, 120, 240, 360, 480, 720 and 1440 minutes) were measured as indicated time points. Experiments were performed at room temperature (25-28°C)

Experiments were repeated as method described above using the different lots of TPDF compared to CPDF.

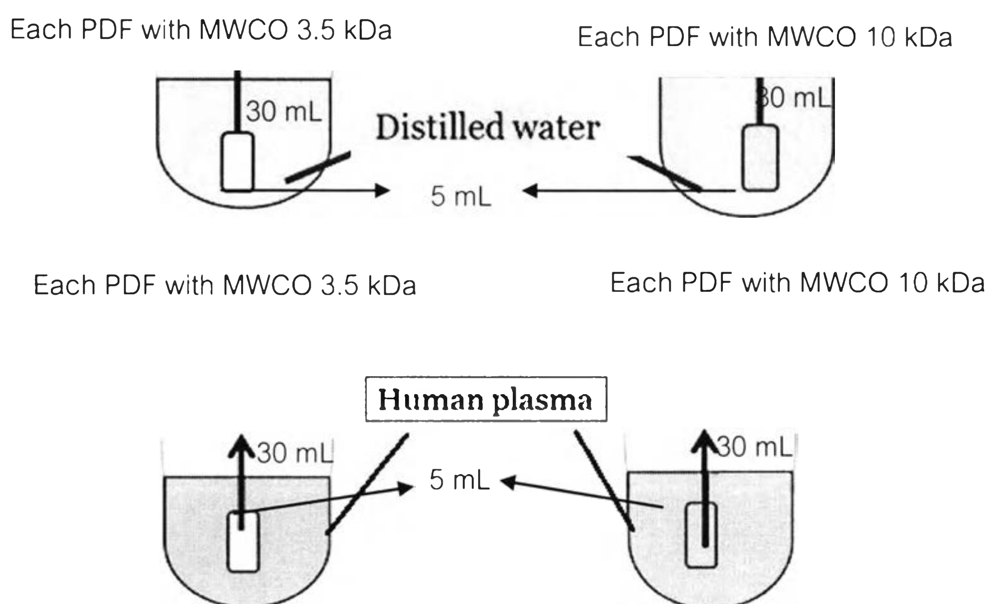


Figure 3.1: PDFs induce water osmosis in water (upper) and plasma (lower) containers

3.4.2 Mechanism of polyglucose based as peritoneal dialysis fluid

It has been previously reported that glucose polymer CPDF functions as a colloid osmotic agent. In theory, crystalloids will diffuse out to the solvent simply. At the same time, the difference between the concentrations will induce a flow of water towards the other side, which has higher crystalloid concentration. The difference between the solute concentrations induces the flow from high solute concentration towards the other side with lower solute concentration, while colloid cannot diffuse out, as simply illustrated in Fig. 3.2.

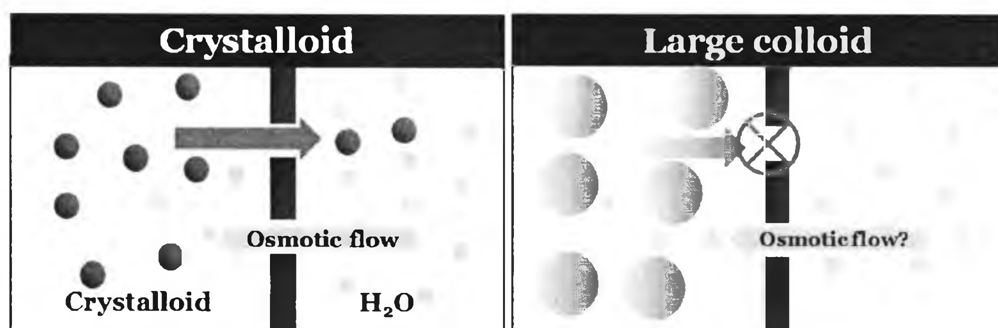


Figure 3.2: Two mechanisms of heterogamous glucose polymer as osmotic agent to induce water flow

However, our believed is that the glucose polymer with heterogeneous molecular weight distribution properties with included small molecules less than 1nm or 1.08kDa, which is in terms of molecular size function, a fraction of glucose polymer with sizes smaller than 1nm it should act like crystalloid.

Besides the colloid osmotic effect, therefore, either TPDF or CPDF should have both crystalloids and colloids in its composition. So we tried to prove the hypothesis of polydispersity glucose polymers with small molecules less than 1.08kDa of CPDF and TPDF have both function colloid and crystalloid.

The aim of the experiment was to prove whether glucose polymer based PDF induce water transport by both colloid and crystalloid osmotic pressure. We are demonstrating the effect of "Crystalloid" mechanism on ultrafiltration induced by both fluids and how that is effective related to the membrane pore sizes.

The experiment is divided into three parts

(1) To evaluate the polyglucose molecules on water transportation using CPDF as a model

First, CPDF was desalted using resin ion exchange. Electrolytic composition was assessed by the ion-selective electrode method (Cobas Integra; Roche Diagnostics, Basel, Switzerland). The osmolality of solutions before and after desalting was determined using a freezing point depression (FPD) osmometer (Advanced® Model 3250, Single-Sample Osmometer, ADVANCED INSTRUMENTS, INC, and Norwood, Massachusetts, USA).

Water osmosis induced by desalted and non-desalted CPDF were performed. Cellophane bags containing desalted CPDF solution were placed in beakers containing distilled water and plasma. 5ml of desalted CPDF were contained in cellophane bags with 3.5kDa and 10kDa MWCOs and the bags were placed in 30ml water containers (as shown at Fig. 3.3) with a slow movement (70rpm). The cellophane bags were weighed at the same dwell time. At five minutes, each bag's mass was weighed as baseline. Then, the mass increase induced by dialysis fluids with different dwell times (30, 60, 120, 240, 360, 480, 720 and 1440 minutes) were measured as indicated time points. Experiments were performed at room temperature (25-28°C)

In addition, the MW distributions of CPDF inside and outside the cellophane bags were determined at the beginning of the experiment and after 24 hours dwelled by HPLC. Gel filtration of HPLC was used to quantify high molecular weight (HMW) fractions.

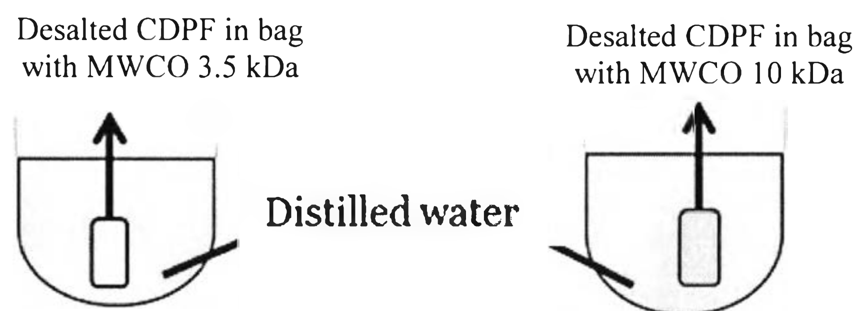


Figure 3.3: Effects of desalted and non desalted CPDF on water osmosis

The same experiment was performed again to examine the efficacy of TPDF to induce water osmosis was performed compared to CPDF and GPDF using the vilified method as same as water osmosis experiments but with the beakers containing blood plasma, as presented in Fig. 3.4. Two different molecular weight cutoffs MWCO 3.5 and MWCO 10kDa containing 5ml of each CPDF were separately placed into 30 ml blood plasma tubes.

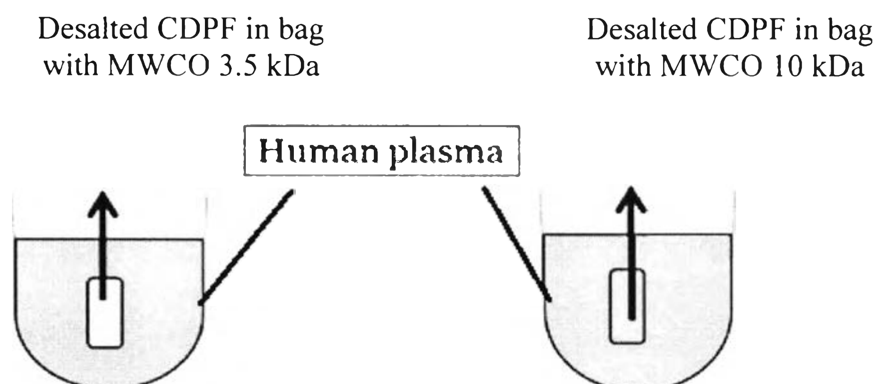


Figure 3.4: Effect of colloid and crystalloid molecules on water osmosis

Calculation and direct examination of mass changes were determined by measuring the difference between the baseline weight of each cellophane bag after being placed in a beaker for 5 minutes (w0) and its weight after each dwelling times interval (wi). The percentage of mass increases was calculated as shown in equation (2). Statistical Analysis with mean values and standard deviation of the percentage of mass changes are assessed.

$$\text{\% Mass Change of Cellophane Bag} = \frac{\text{Weigh}_{(t)} - \text{Weight}_{(to)}}{\text{Weight}_{(to)}} \times 100 \qquad (2)$$

(2) To throw light on properties of high and low molecular weight effect on water osmosis using corn-based as osmotic agents was performed. The MW distributions of CPDF inside and outside the cellophane bags were determined at the beginning of the experiment and after 24 hours dwelled as above HPLC analysis method.

HPLC (Size exclusion chromatograph) was performed with a Waters Corpoation, MS, USA equipped with refractive index detector (Water 410 Differential Refractometer). The column used was a Ultrahydrogel™Linear (300mm × 7.8mm id × 2) connected to Ultrahydrogel 250 two columns (Water Corporation, MS, USA). The mobile phase was non-ionized water with resistance 18.2 mΩ after filtrated through 0.45 um and degas by Branson Ultrasonic 2210. The column temperature was 40 °C. The flow-rate was 0.9

mL/min and the injection volume was 20μL. It will take about 45 minutes for 1 run of sample. Chromatogram will record retention time (RT) and area under curve of the tests compared to the molecular weight standardized calibration curve. Molecular weight by weight (Mw) was calculated

(3) To prove *in vitro* ultrafiltration, a computer simulation using MATLAB software was applied. A mathematical model was created to explain water transportation induced by CPDF as a model compared to experiment model.

A model referred to Rippe *et al.*, (2002) was used, but with only one pore size, which we will call the effective pore size. These are the transport equations that we used. Ultrafiltration between TPDF, CPDF and GPDF was compared to previous studies [38, 217].

Transport Equations **(1)**

Hydrostatic Pressure   Osmosis   Lymphatic Flow

$$\frac{dV_D}{dt} = Q_U = L_p A(\Delta P - \sum \sigma_{i,crystalloid} \Delta\pi_{crystalloid} - \sum \sigma_{i,colloid} \Delta\pi_{i,colloid}) - Q_L$$

Diffusion   Convection

$$\frac{dV_{D,i} C_{D,i}}{dt} = KA(C_{B,i} - C_{D,i}) + WQ_U(C_{B,i}) - Q_L C_{D,i}$$

Van's Hoff equation : $\Delta\pi_i = RT(C_{B,i} - C_{D,i})$

L_p = Ultrafiltration Coefficient

K = Mass Transfer Coefficient

W = Convective Hindrance Factor

The hydrostatic pressure and lymphatic reabsorption are not presented, so the flow is induced by osmosis alone, while solute concentration is governed by diffusion and convection.

3.5 Statistical analysis

The SPSS version 16.0 package was used. Pair and unpaired student t-test were used appropriated used. Nonparametric tests: Differences between groups were analyzed using Mann-Whitney U test, different times in the same group was analyzed using Wilcoxon signed-rank test. Spearman rank-order correlation was used to determine the correlation between two variables. All values are reported as mean ± standard error (SE), *p values* less than 0.05 is considered as statistically significant.