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14. ABSTRACT

The Porphysome is an all-organic, biodegradable and biocompatible nanomaterial that exhibits multiple structurally depend modes-of-action emerging from a single, easily synthesized building block: the Pyropheophorbide—lipid. With over 40 research articles on its biophotonic, radiologic, and therapeutic applications in cancer imaging and treatment, the Porphysome is an ideal drug candidate for further clinical translation. Herein are reported recent progresses towards the production of Porphysomes with scalable cGMP manufacture, the development of a kit to prepare radio-pharmaceutical Porphysomes with PET radionuclide 64-Copper of quality suitable for human administration, and the preliminary pharmacology and toxicology profile of radio-pharmaceutical Porphysome in preclinical models. Radiation dosimetry and acute toxicology remain outstanding tasks before the Porphysome may proceed towards CTA submission with Health Canada for first-in-human studies.

15. SUBJECT TERMS

Nanomedicines; Good Manufacturing Practice; Radio-pharmaceutical kit; Radio-labelling; Positron Emission Tomography; Pharmacology and toxicology; Clinical Trial Application

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Introduction:
Nanomedicines have already established a tangible presence in today's market for cancer therapies with over 9 United States Food and Drug Administration (US FDA) approved nanomedicines and a further 90 under clinical investigation for oncology [1]. By exploiting recent discoveries in nanotechnology and tumour biology, nanomedicine design has yielded increasingly sophisticated agents consolidating multiple modes of action, such as imaging and therapeutic capabilities, into "all-in-one" platforms. However, the rise of multifarious nanomedicines has also magnified practical limitations hindering the clinical translation of complex agents: multi-component formulations are challenging to scale-up manufacture, may rely upon contested intellectual property, and demand an onerous regulatory pathway. Consequently, as many as 85% of promising nanomedicines for oncology ultimately fail to advance beyond academic investigation [2].

The first barrier to the translational success of nanomedicines is the ability to produce agents in accordance with quality control systems such as current Good Manufacturing Practices (cGMP) [3] and on a scale necessary to complete preclinical pharmacology and toxicology, and sustain early Phase trials. This means that the usually complex chemistry of nanomedicines need to be thoroughly described in Standard Operating Procedures (SOPs) and followed through step-by-step by preparers in a cGMP certified site under aseptic conditions. It also entails the development of analytical methods capable of examining concentration and purities of each component of the nanoproduct before product release. The manufacturing process of nanomedicines needs to be sufficiently robust and reliable for producing the same product time after time with the intended physicochemical characteristics, biological behaviors, and pharmacological profiles. Thus the more complex the product, the more difficult it is to fulfil the requirements for cGMP scalability.

The second barrier to translation is the dose [4]: the dose required to obtain the desired effect (effective dose), the dose at which off-target toxicity is observed (maximum tolerated dose), and the ratio of the two known as the therapeutic index. Incorporating therapeutic agents into nanomaterials potentially offers superior accumulation in target organ(s) and longer circulation times relative to free-drug solutions, thus reducing the minimum therapeutically effective dose. However, nanomaterials themselves are not toxicologically inert: depending on the size, material chemistry, and surface modifications, nanomaterials may accumulate in the liver, lungs, and spleen, while hampering organ function, accelerating inflammation, and triggering immune responses [4]. Another dose consideration is how the formulation might be administered in a clinical setting (e.g., infusion volume and frequency). Demonstrating a superior safety profile in preclinical models is only the first step towards finding dosages for first-in-man studies, dose assessment and possible dose expansion in patients' needs to be understood and investigated in a preclinical context as well.

A new approach towards designing nanomedicines seeks to address, overcome or avoid entirely the translational hurdles identified above from the point of inception with a "bottom-up" design approach. Examples of this new design strategy can be recognized in the art of "one-for-all" nanomedicines wherein multiple material functionalities emerge from a single, easily synthesized building block as opposed to a combination of clinically untested products [5]. The Porphysome is an exemplary "one-for-all" nanomaterial with over 40 research articles on its biophotonic, radiologic, and therapeutic applications in cancer imaging and treatment (*Figure 1*). Herein are reported recent progresses towards the production of Porphysomes with scalable cGMP manufacture, the development of a kit to prepare radio-pharmaceutical Porphysomes with Positron Emission Tomography (PET) radionuclide 64-Copper (^{64}Cu) of a quality suitable for human administration, and the preliminary pharmacology and toxicology profile of radio-labelled Porphysome in preclinical models.

Body:
Unilamellar nanovesicles self-assembled ex vivo (Figure 2).
The Porphysome is an all-organic nanomaterial formulated with proprietary *pyro*pheophorbide-α–lysophosphatidylcholine conjugate (pyro–lipid), cholesterol, and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DSPE-mPEG$_{2000}$) in molar ratio 55.0, 40.0, and 5.0, respectively. Cholesterol and DSPE-mPEG$_{2000}$ are clinically approved for generic pharmaceutical

preparations, whereas pyro–lipid an investigational imaging agent which has demonstrated biocompatibility, biodegradability, and low toxicity in preclinical models [6]. The preparation of Porphysomes follows a conventional liposomal preparation procedure (see *Figure 3*) with high-pressure extrusion through sequentially decreasing filter pore sizes to create spherical, unilamellar nanovesicles with a monodisperse, kinetically tailorable size distribution. Porphysomes prepared in Phosphate Buffered Saline (PBS), pH 7.4, demonstrate remarkable shelf-life stability at 4 °C (39 °F) with negligible changes in their hydrodynamic diameter (*Figure 4A*), polydispersity index (PDI) (*Figure 4B*), pyro–lipid concentration (*Figure 4C*), surface/zeta-potential (*Figure 4D*), and fluorescence self-quenching (*Figure 4E*) over a 6-month period.

cGMP manufacture of Porphysomes suitable for human administration.
The small scale manufacture of Porphysomes is fully described in forty-two (42) Standard Operating Procedures (SOPs) (*Figure 5*) and is performed in a specialized laboratory meeting the requirements of current Good Manufacturing Practice (cGMP). All the ingredients used throughout the formulation and manufacture of Porphysomes are of a suitable United States Pharmacopoeia (USP) or National Formulary (NF) grade purchased through commercial suppliers. Certificates Of Analysis (COAs) are obtained from suppliers and are added to in-house production and quality control records for each lot of Porphysomes manufactured. Products for which no pharmacopoeial grade is commercially available (e.g., pyro–lipid), analytical techniques are employed to characterize the material and establish the purity of the material (usually > 95.0% required) and levels of trace impurities as per United States Food and Drug Administration (US FDA) guidance. Wherever necessary quality specifications, parameters, and assays for USP non-sterile pharmaceutical compounding have been incorporated into the manufacture of Porphysomes in order to fulfil quality and safety requirements for human administration; these specifications include requirements/restrictions on the volume, pH, appearance, sterility, bacterial endotoxins, product label, etc. of the final material. For instance, the pyrogenicity and sterility of newly manufactured Porphysomes are evaluated with USP Bacterial Endotoxin Test and USP Sterility Test protocols, respectively. Only upon satisfying all the prescribed quality specifications and acceptance criteria is a batch/lot of Porphysomes released for further manufacture.

Radio-pharmaceutical kit to prepare Porphysomes (Figure 6).
A radio-pharmaceutical kit to prepare Porphysomes intended for radio-labelling with Positron Emission Tomography (PET) radionuclide 64-Copper (^{64}Cu) has been developed. The kit consists of a single unit-dose vial containing the non-radionuclide Porphysomes. Each kit consists of 0.494 mg of pyro–lipid, 0.137 mg of cholesterol, 0.124 mg of DSPE-mPEG$_{2000}$ and approximately than 10.3 mg of buffer salts in 0.975 mL of Phosphate Buffered Saline (PBS), pH 7.4. Trace metals (e.g., copper, nickel, etc.) and other impurities have been assayed previously during the cGMP manufacture of the Porphysomes and are recorded in the quality control documentation accompanying each released batch/lot of Porphysomes. No additional preservatives are added to the kit. The expiry period (shelf-life) of each kit is 6 months from the date the Porphysome batch/lot was released when stored under the prescribed conditions and left undisturbed; thus the sterility and apyrogenicity, and material characteristics of the Porphysomes (e.g., hydrodynamic diameter, storage concentration, etc.) characterized at the time of bath/lot release are guaranteed for not more than 6 months after release.

The radionuclide 64-copper (II) chloride diluted in radio-labelling buffer 0.1 M Ammonium Acetate Buffer (NH_4OAc Buffer), pH 5.5, is added to the vial containing Porphysomes in order to prepare the radio-pharmaceutical (^{64}Cu-Porphysome) for use (*Figure 7*). Specifically, to a unit-dose vial containing Porphysomes, approximately 203.5–240.5 MBq (5.5–6.5 mCi) of ^{64}Cu activity prepared in 0.0975 mL of NH_4OAc Buffer is added. The “on-pot” radio-labelling of the Porphysomes requires heating to at least 60°C (140°F) for at least 30 minutes. Instant Thin-Layer Chromatography (iTLC) is used to assess the radio-chemical purity of the resulting ^{64}Cu-Porphysome with an acceptance criterion of not less than 90%. The above described procedure for radio-labelling Porphysomes is robust and highly efficient (> 90% radio-labelling efficiency) owing to the strong and stable chelation chemistry of the *pyro*pheophorbide moiety in pyro–lipid for ^{64}Cu [7]. Higher radio-labelling efficiencies are desirable for obviating the need for post-labelling purification for the ^{64}Cu-Porphysome from free ^{64}Cu prior to patient administration. The specific activity of ^{64}Cu-Porphysomes of up to 1,3321 MBq/μmol

(approximately 1,036 TBq/μmol per nanovesicle) is the greatest reported for an all-organic nanovesicle [7]. The final radio-pharmaceutical (^{64}Cu-Porphysome) is terminally sterilized by filtration through a 0.22-μm syringe filter into a supplied vial containing sterile PBS, pH 7.4, in order to restore the osmolality and neutrality of the injectable ^{64}Cu-Porphysome solution. Thus the radio-pharmaceutical kit to prepare Porphysomes prepares a single injection of sterile ^{64}Cu-Porphysomes with ~185 MBq (~5.0 mCi) of radioactivity in ~5.0 mL (as in *Figure 7*). The expiry of the prepared ^{64}Cu-Porphysomes is 24 hours (i.e., two physical half-lives of ^{64}Cu) after radio-labelling when stored at room temperature 25°C (77°F).

Preclinical pharmacology and toxicology of Porphysomes

The biodistribution of ^{64}Cu-Porphysomes to the tissues of healthy, immuno-competent Copenhagen rats was evaluated at 6, 12, 24 and 48 hours' post-intravenous administration. Five male rats (277 ± 39 g) receiving 56.98 ± 8.14 MBq (1.54 ± 0.22 mCi) of ^{64}Cu-Porphysomes with 4.21 ± 0.13 mg of pyro–lipid per kg of weight are sacrificed at each timepoint. Organs are removed for semi-quantification of *pyro*pheophorbide fluorescence (*data not shown*) with *ex vivo* hyperspectral imaging and for quantification of ^{64}Cu (*Figure 8*) with scintillation counting. Large and persistent accumulation of ^{64}Cu activity in the liver, kidneys, spleen, and lungs over 48 hours post intravenous administration is indicative of classical mononuclear phagocytic system (MPS) recognition and uptake [8]. The pharmacokinetics of ^{64}Cu-Porphysomes are similarly evaluated in healthy, immuno-competent Copenhagen rats with repeated blood sampling at nine timepoints over 30 hours' post-intravenous administration (*Figure 9*). Four male rats (268 ± 9.8 g) receiving 85.1 ± 14.8 MBq (2.3 ± 0.4 mCi) of ^{64}Cu-Porphysomes with 4.63 mg of pyro–lipid per kg of weight had between 100–300 μL of blood removed from a jugular vein catheter at each timepoint for quantification of ^{64}Cu with scintillation counting. Fitting the blood clearance of ^{64}Cu activity with a two phase decay (i.e., two-compartment model) yields a $t_{½,\alpha}$=0.6036 hour distributive phase and a $t_{½,\beta}$=10.73 hour elimination phase. Thus the effective half-life of the ^{64}Cu-Porphysome is $t_{½,eff}$=5.81 hours. The linearity of the ^{64}Cu-Porphysome pharmacokinetics is an outstanding task. The above data will be used to predict the radiation absorbed doses in humans (i.e., radiation dosimetry) using the Organ Level Internal Dose Assessment (OLINDA) software.

Key Research Accomplishments:

- Standard Operating Procedures (SOPs) developed for the scalable manufacture of Porphysomes and meeting the requirements of current Good Manufacturing Practice (cGMP).
- Development of a kit to prepare a unit dose of Porphysomes radio-labelled with Positron Emission Tomography (PET) radionuclide 64-Copper (^{64}Cu) and intended for intravenous (parenteral) administration in humans.
- Biodistribution and pharmacokinetics of ^{64}Cu-Porphysomes was quantified in healthy immunocompetent rats with *ex vivo* scintillation counting. Radiation dosimetry and acute toxicology remain outstanding tasks to be completed.

Reportable Outcomes:

- Manuscript: Valic, M.S., Zheng, G. *Rethinking translational nanomedicine: Insights from the "bottom-up" design of the Porphysome for guiding the clinical development of imageable nanomaterials.* Curr Opin Chem Biol. (2016), 33(August 2016):xxx-xxx.
- Abstract: Valic, M.S., Ye, T., Zhang, C., Jiang, W., Chen, J., Bernardini, M.Q., Zheng, G., "All-organic nanovesicles for multimodal PET/CT and optical fluorescence assessment of lymphatic tumour disseminations." Oral presentation, American Society of Photobiology Conference 2016, May 2016, Tampa, USA.
- Abstract: Valic, M.S., Ye, T., Zhang, C., Jiang, W., Chen, J., Bernardini, M.Q., Zheng, G., "Targeted all-organic nanovesicles for multimodal PET/CT and optical fluorescence assessment of lymphatic disseminations in gynaecological cancers." Poster presentation, Princess Margaret Cancer Centre Personalizing Cancer Medicine in 2016, Feb. 2016, Toronto, Canada.
- Abstract: Valic, M.S., Jiang, W., Chen, J., Bernardini, M.Q., Zheng, G., "Targeted all-organic nanovesicles for multimodal PET/CT and optical fluorescence assessment of lymphatic disseminations

in gynaecologic cancers." Oral presentation, 14th International Summer School on Biocomplexity, Biodesign and Bioinnovation: from Gene to System, Jun. 2015, İzmir, Turkey.

- Abstract: Valic, M.S., Jiang, W., Chen, J., Bernardini, M.Q., Zheng, G., "Targeted all-organic nanovesicles for multimodal PET/CT and optical fluorescence assessment of lymphatic disseminations in gynaecologic cancers: A kit to prepare parenteral injections for a "first-in-woman" clinical study." Oral presentation, World Congress on Medical Physics & Biomedical Engineering, Jun. 2015, Toronto, Canada.
- Abstract: Valic, M.S., Jiang, W., Chen, J., Bernardini, M.Q., Zheng, G., "Targeted all-organic nanovesicles for multimodal PET/CT and optical fluorescence assessment of lymphatic disseminations in gynaecologic cancers: A radio-pharmaceutical kit to prepare parenteral injections for a "first-in-woman" clinical study." Poster presentation, Ontario Cancer Research Institute Scientific Day 2015, May 2015, Toronto, Canada.

Outstanding Tasks:

The final step in clinical translation of the Porphysome platform is regulatory agency submission. In Canada, a Clinical Trial Application (CTA) must be submitted to Health Canada—analogous to an Investigational New Drug (IND) submission to the US FDA. The CTA for a Phase I trial in Canada requires three supporting modules: (i) chemistry & manufacturing, (ii) preclinical pharmacology and toxicology, and (iii) clinical trial protocols, investigational brochure and informed consent. The work reported above goes towards satisfying the first of these two modules; specifically addressing the manufacturing and pharmacology of the Porphysomes. Outstanding tasks include the procurement of pyro–lipid from a cGMP manufacturer and experimental determination of the radiation dosimetry and acute toxicology of radio-pharmaceutical ^{64}Cu-Porphysomes. Progress towards the third module of the CTA is on-going in parallel with the work reported above (*not discussed*).

Conclusion:

Measurable progresses have been made towards the clinical translation of the Porphysome platform including (1) the development of Standard Operating Procedures (SOPs) with the scalable manufacture of Porphysomes meeting the requirements of current Good Manufacturing Practice (cGMP), (2) the development of a kit to prepare radio-pharmaceutical Porphysomes labelled with Positron Emission Tomography (PET) radionuclide 64-Copper (^{64}Cu) of a quality suitable for human administration, and (3) determination of the pharmacology and toxicology profiles of radio-pharmaceutical Porphysomes in preclinical models. Radiation dosimetry and acute toxicology remain outstanding tasks before the Porphysome platform may proceed towards Clinical Trial Application (CTA) submission with Health Canada for first-in-human studies.

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Appendices:

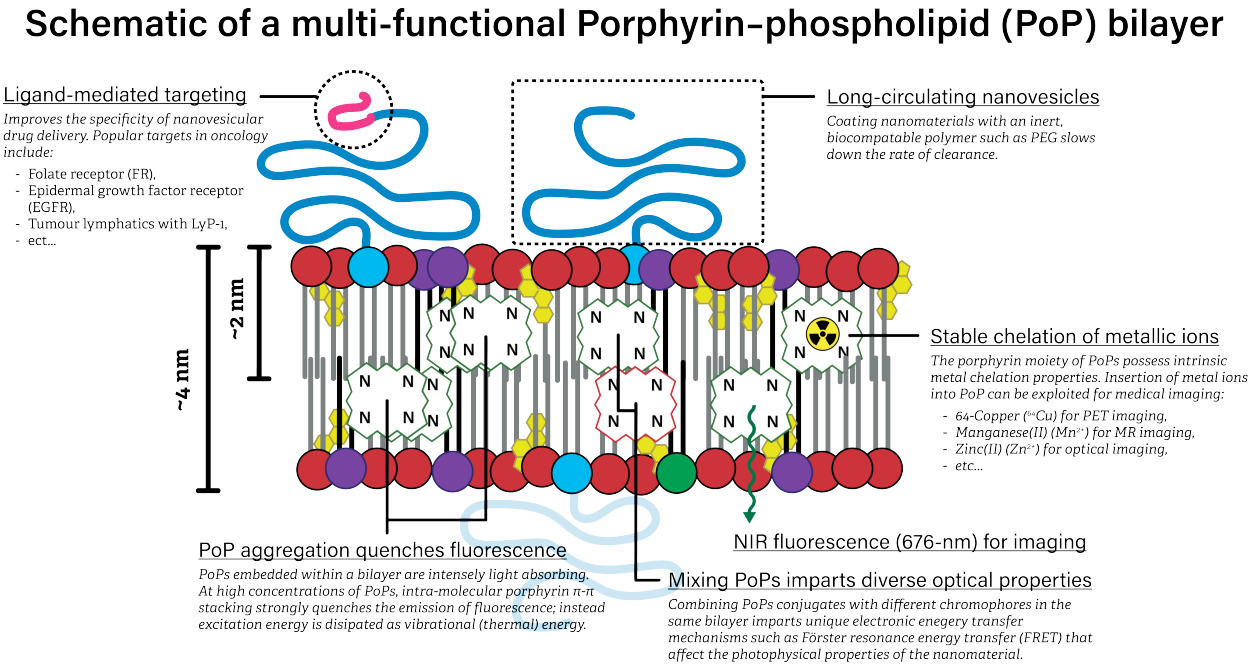

Figure 1: Overview of the multi-functional potential of the porphyrinoid–phospholipid (PoP) bilayered structures: Targeting these nanomaterials by functionalizing with receptor ligands, antibodies, etc., chelating paramagnetic metal ions with the porphyrin building-blocks for MR imaging contrast, exploiting Porphysomes high-payload chelating abilities to deliver radiotherapeutics, and exploiting structurally dependent *modes-of-action* such as the strong absorbance of porphyrins for photothermal therapy with highly quenched, intact PoP bilayers, and photodynamic therapy with PoP monomers following dissociation of the nanostructure.

Pyropheophorbide-lipid Porphysome

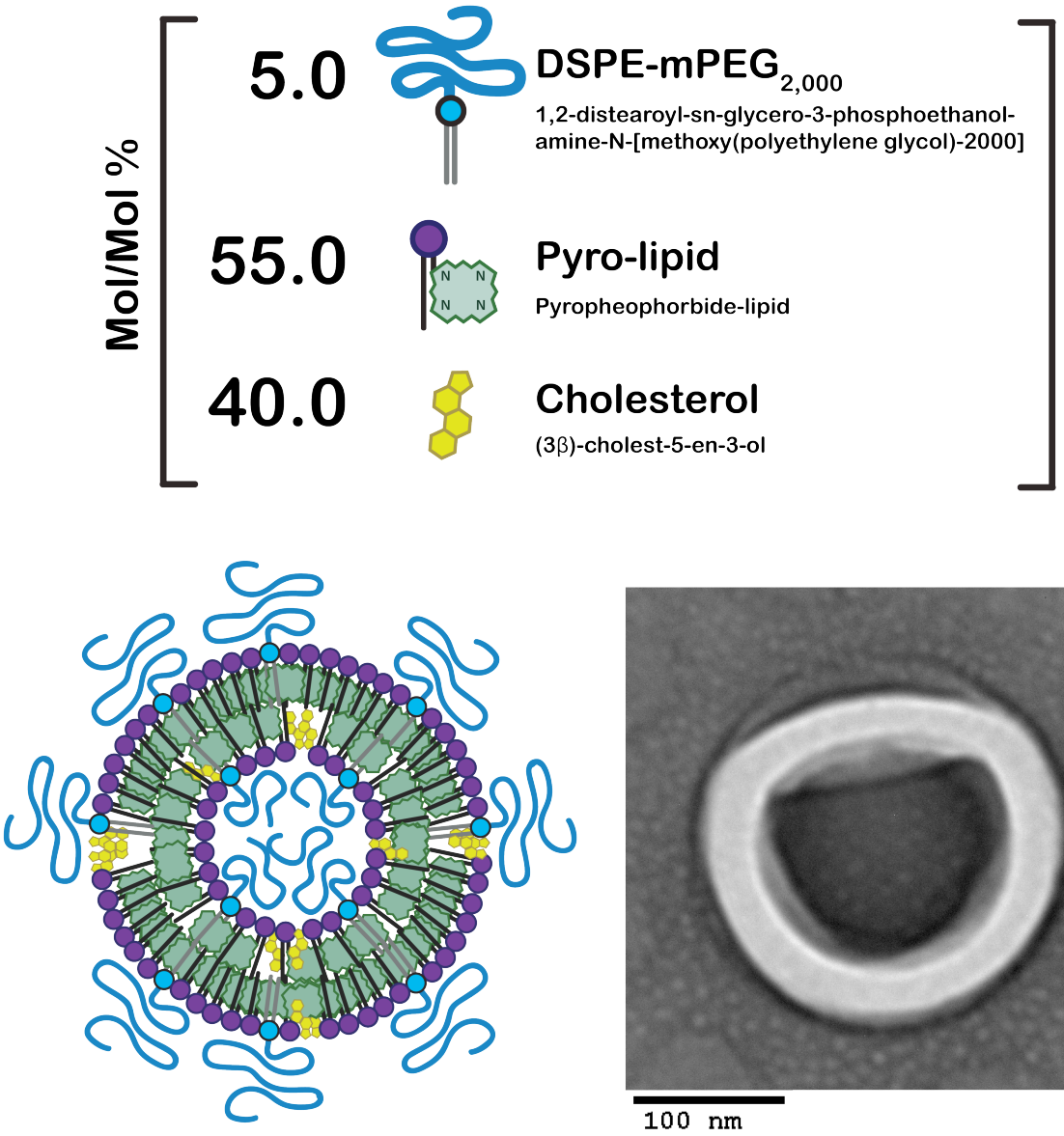

Figure 2: The *pyro*pheophrobide–lipid (pyro–lipid) Porphysome, a unilamellar nanovesicles self-assembled *ex vivo*.

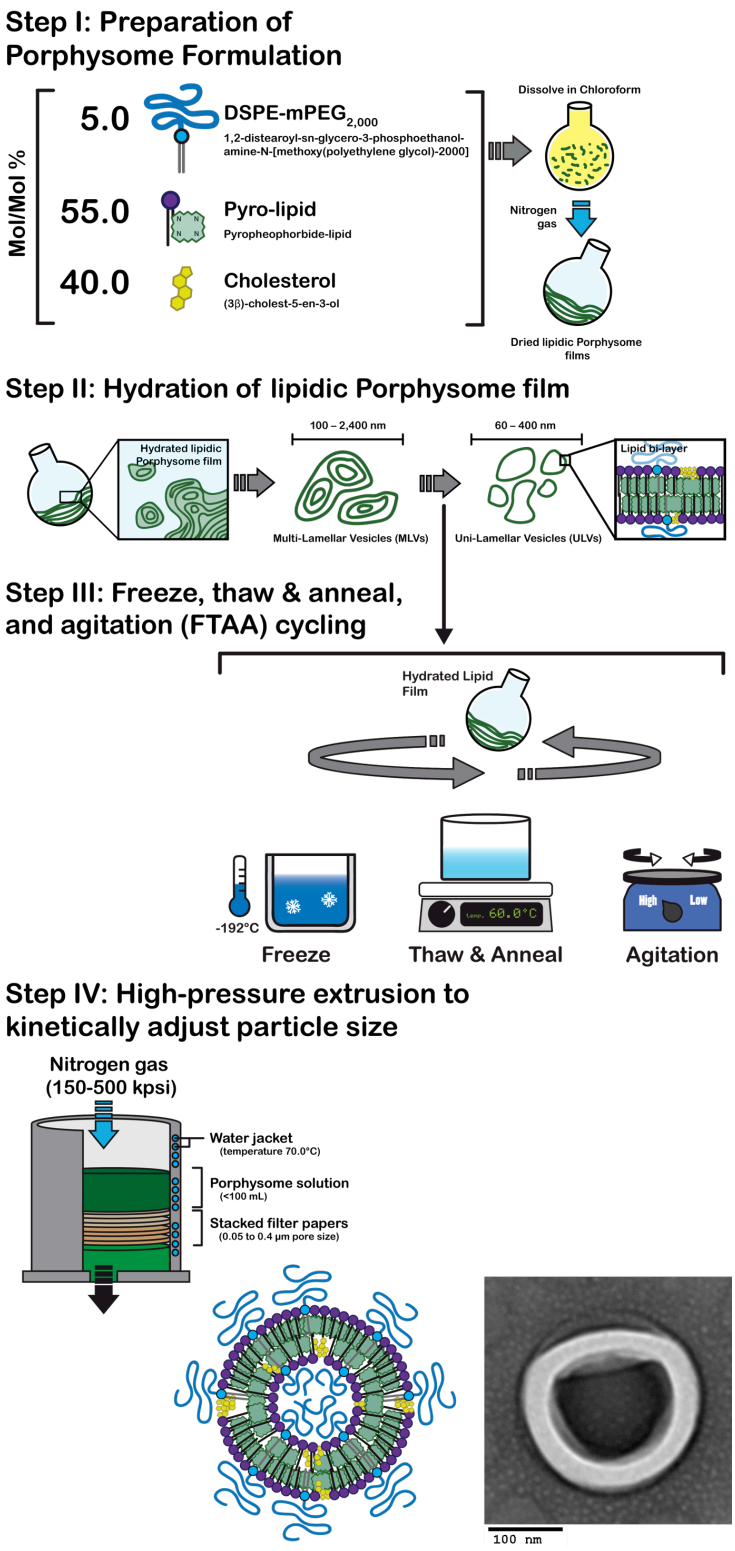

Figure 3: Schematic of a conventional liposomal preparation procedure with high-pressure extrusion adopted for the manufacture of Porphysomes.

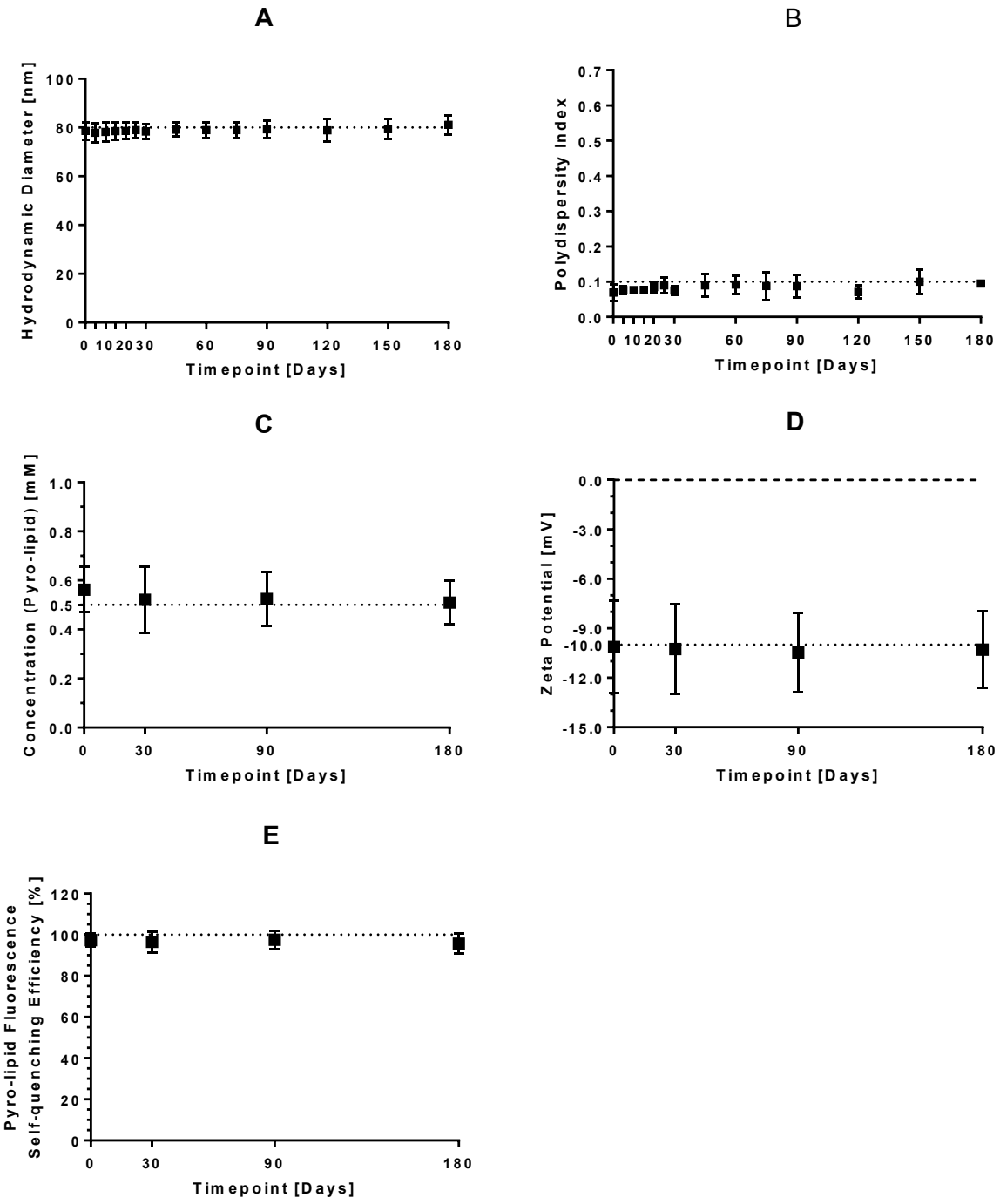

Figure 4: Shelf-life stability of Porphysomes prepared in Phosphate Buffered Saline (PBS), pH 7.4, at 4 °C (39 °F) over a 6-month period. (*A*) hydrodynamic diameter and (*B*) polydispersity index (PDI) measure with photon correlation spectroscopy (PCS), (*C*) pyro–lipid concentration measured with absorbance spectrophotometry, (*D*) surface/zeta-potential with Phase Analysis Light Scattering (PALS), and (*E*) fluorescence self-quenching with fluorescence spectroscopy. Each data point represents the mean ± 1 standard deviation with n=5 unless indicated otherwise.

Raw Material Specifications (SOP-RM-###)	
SOP No.	*Title*
SOP-RM-001	Acetic Acid, Glacial
SOP-RM-002	Ammonium Acetate
SOP-RM-003	Argon
SOP-RM-004	Cation-Exchange Resin, Styrene-Divinylbenzene
SOP-RM-005	Chloroform
SOP-RM-006	Cholesterol
SOP-RM-007	64-Copper(II) Chloride, Aqueous
SOP-RM-008	Depyrogenated Sterile Empty Vials, 2-mL
SOP-RM-009	Depyrogenated Sterile Empty Vials, 10-mL
SOP-RM-010	DSPE-mPEG(2000)
SOP-RM-011	DSPE-PEG(2000)-Folate
SOP-RM-012	Hydrochloric Acid
SOP-RM-013	Nitrogen
SOP-RM-014	Potassium Chloride
SOP-RM-015	Potassium Phosphate, Monobasic
SOP-RM-016	Pyropheophorbide-lipid
SOP-RM-017	Sodium Chloride
SOP-RM-018	Sodium Hydroxide
SOP-RM-019	Sodium Phosphate, Dibasic, Heptahydrate
SOP-RM-020	Sterile Disposable Syringe Filters, 0.2-µm
SOP-RM-021	Sterile Vacuum Filtration System, 0.2-µm
SOP-RM-022	Sterile Water for Injection

Intermediate Specifications (SOP-INT-###)	
SOP No.	*Title*
SOP-INT-001	Preparation of 0.1 M Ammonium Acetate Buffer pH 5.5
SOP-INT-002	Preparation of Phosphate Buffered Saline pH 7.4
SOP-INT-003	Preparation of Phosphate Buffer with EDTA for Radio-chromatography
SOP-INT-004	Preparation of Dried Porphysome Lipidic Film
SOP-INT-005	Preparation of Dried Porphysome Lipidic Film
SOP-INT-006	Preparation of Unilamellar Porphysome Nanovesicles
SOP-INT-007	Preparation of Radio-Pharmaceutical Kit of Porphysomes

Test Protocols (SOP-TP-###)	
SOP No.	*Title*
SOP-TP-001	Absorption Spectrophotometry
SOP-TP-002	Appearance
SOP-TP-003	Assignment of Lot Number & Expiry
SOP-TP-004	Bacterial Endotoxins Test
SOP-TP-005	Bubble Point Test
SOP-TP-006	Fluorescence spectoscopy
SOP-TP-007	Label Check
SOP-TP-008	Liquid Chromatography-Mass Spectrometry
SOP-TP-009	pH Determination
SOP-TP-010	Photon Correlation Spectroscopy
SOP-TP-011	Proton Nuclear Magnetic Resonance
SOP-TP-012	Radiochemical Purity
SOP-TP-013	Sterility

Figure 5: Compete list of the Standard Operating Procedures (SOPs) describing in full detail the manufacture of the Porphysome meeting the requirements of current Good Manufacturing Practice (cGMP).

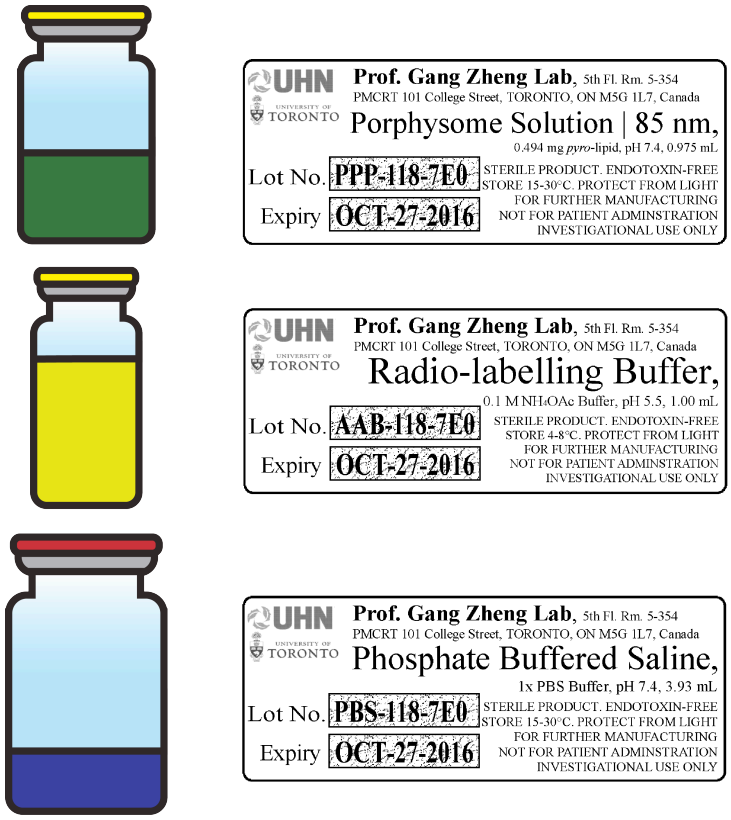

Figure 6: Schematic of the radio-pharmaceutical kit to prepare a single dose of Porphysomes radio-labelled with 185 MBq (5 mCi) of Positron Emission Tomography (PET) radionuclide 64-Copper (^{64}Cu). The kit is composed of a unit-dose vial of Porphysomes (*top*), radio-labelling buffer—0.1 M ammonium acetate buffer, pH 5.5 (*middle*), and phosphate buffered saline, pH 7.4 (*bottom*).

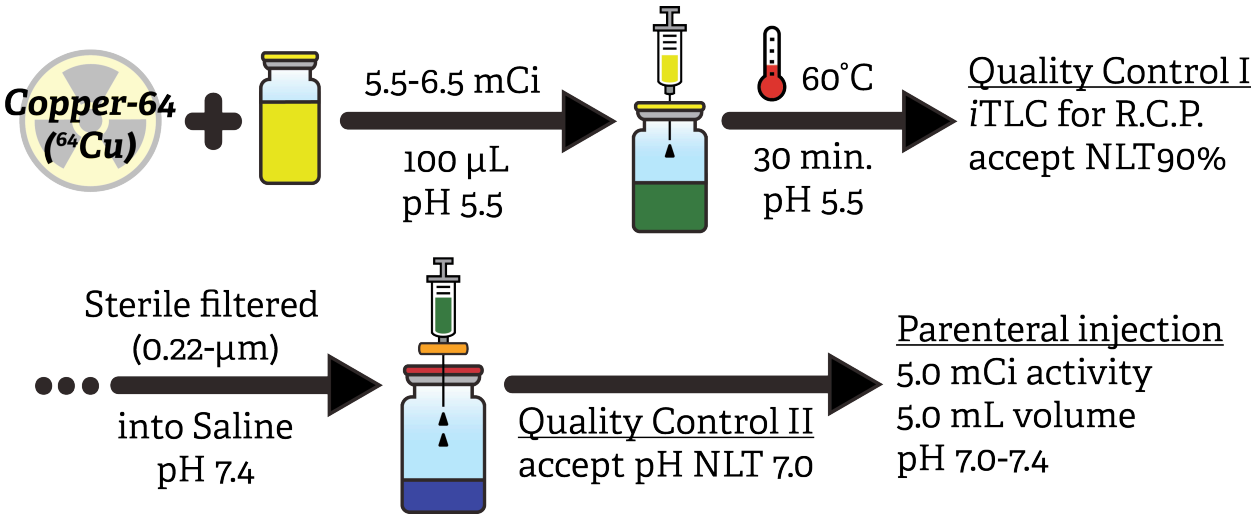

Figure 7: Workflow of the radio-labelling procedure for the kit to prepare Porphysomes.

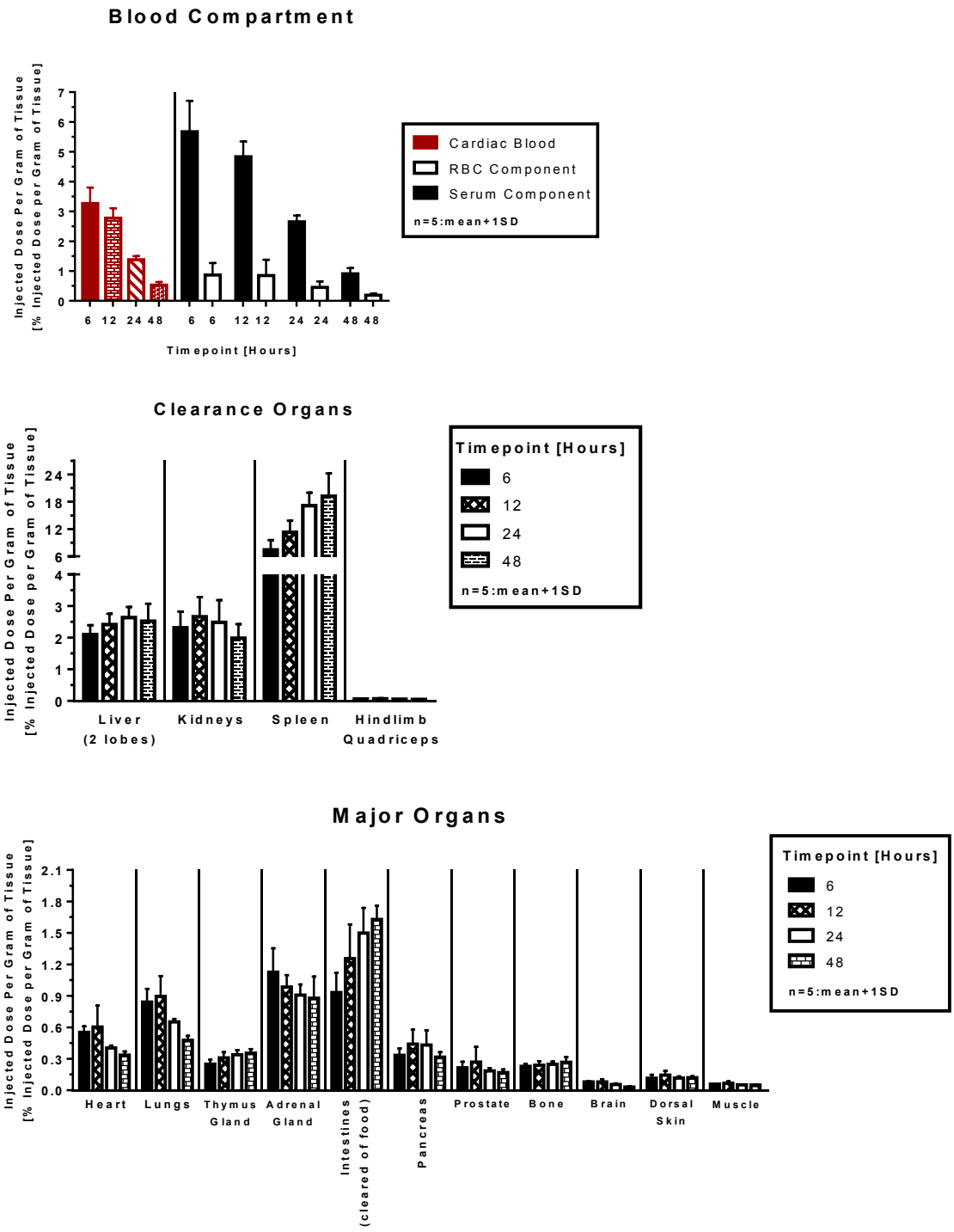

Figure 8: Biodistribution of ^{64}Cu-Porphysomes in the tissues of healthy, immunocompetent rats receiving intravenously administered dose of 56.98 ± 8.14 MBq (1.54 ± 0.22 mCi) with 4.21 ± 0.13 mg of pyro–lipid per kg of weight. ^{64}Cu was quantified with *ex vivo* scintillation counting. Each data point represents the mean + 1 standard deviation with n=5 unless indicated otherwise.

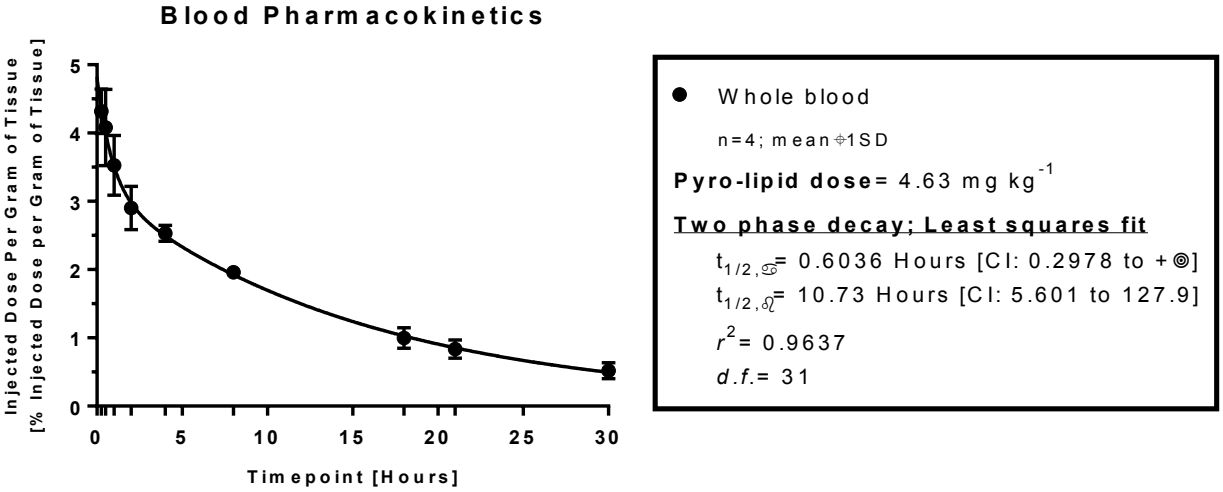

Figure 9: Pharmacokinetics of ^{64}Cu-Porphysomes in healthy, immunocompetent rats receiving intravenously administered dose of 85.1 ± 14.8 MBq (2.3 ± 0.4 mCi) with 4.63 mg of pyro–lipid per kg of weight. ^{64}Cu was quantified with *ex vivo* scintillation counting. Each data point represents the mean ± 1 standard deviation with n=4 unless indicated otherwise.