Critical Process Parameters for The Preparation of Amphotericin B Liposomes

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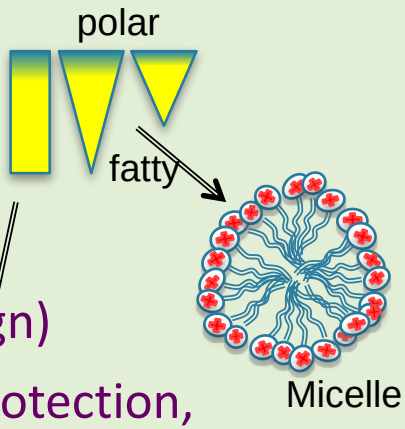
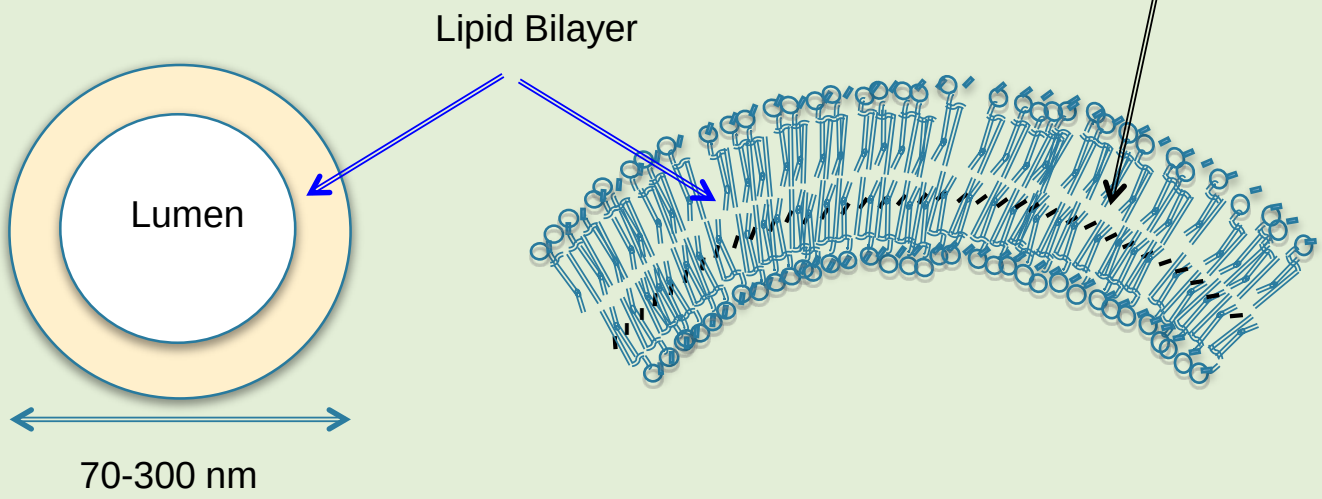
Liposomal Assembly

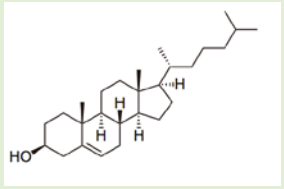
- Based on Amphiphiles (e.g. phospholipids)
- Hydrophilic (Polar), Hydrophobic (Fatty) Groups
- Type of Assemblies (micelles, tubular micelles, bilayers)
- Features (enclosed space, protection, hydrophobic core, surface boundary, modifiable surface, amenable for design)
- Pharmaceutical relevance (half-life, tissue distribution, protection, controlled release, targeting, etc)



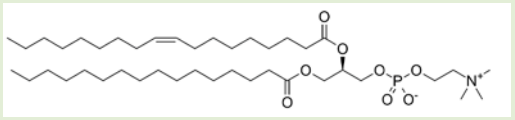


Liposome Composition, Variability and Issues

- Phospholipids
  - Bulk of the membrane bilayer
  - Charge of the surface (head group)
  - Rigidity (Fatty acyls)
  - Liquid or gel phase (saturated FA)
  - Homogeneity
- Cholesterol
  - Invariable element
  - Increasses viscosity
  - Stability/Permeability
  - interact with PL



Cholesterol



Phosphatidylcholine

- Issues with PL's (CMC)
  - PL Purity
  - Sourcing
  - Sterilization
  - Stability
  - Oxidation
  - Encapsulation
  - Leakage

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Examples of Approved Liposomal Drugs

API	Product	Lipids	Route	Benefit	Disease
Amphotericin	Ambisome (1990 EU, 1997 US)	HSPC, DSPG, Chol	IV, Daily	Reduced toxicity	Fungal infection
Doxorubicin	Myocet (2000 EU)	EPC, Chol	IV, Once/3 weeks	Reduced toxicity	Metastatic breast cancer
	Doxil (1995, 1999)	HSPC, Chol, DSPE-PEG2000	IV, Once/2-4 weeks	Extended exposure	Kaposi's sarcoma; ovarian cancer; multiple myeloma
	Lipodox (2013)	Bio-equiv. to Doxil			
Daunorubicin	DaunoXome (1996)	DSPC, Chol	IV, Once/2 weeks	Reduced toxicity	Blood cancer
Verteporfin	Visudyne (2000)	DMPC, EPG	IV, Single dose	Pref. site delivery	Wet macular degeneration (PD therapy)
Cytarabine	Depocyte (1999, 2007)	DOPC, DPPG, Chol, Triolein	Intrathecal, Once/2 weeks	Extended exposure	Neoplastic meningitis
Morphine	DepoDur (2004)	DOPC, DPPG, Chol, Triolein	Epidural, Once/48 h	Extended exposure	Post surgery pain
Vincristine	Marqibo (2012)	Egg Sphingo-myelin, Chol	IV, Once/ 1 week	Extended exposure	Leukemia
Irinotecan	Onivyde(2015)	DSPC, Chol, DSPE-PEG2000	IV, Once/2 weeks	Reduced toxicity?	Pancreatic cancer

Liposomal Drugs Pipeline (Selected)

API	Product	Lipids	Route	Disease
Paclitaxel	EndoTAG-1	DOTAP, DOPC	IV	Breast, pancreatic cancer
Paclitaxel	LEP-ETU	EPC, Cardiolipin, Chol	IV	Breast cancer
Cisplatin	Lipoplatin	SPC, DPPG, Chol, DSPE-mPEG	IV	Pancreatic cancer
Lurtotecan	OSI-211	HSPC, Chol	IV	Ovarian, head and neck cancer
Amikacin	Arikace	DPPC, Chol	Aerosol	Lung Infection
BLP25 Lipopeptide, Monophosphoryl Lipid A	Stimuvax	DMPG, DPPC, Chol	SQ	NSCLC
Annamycin		DSPC, DSPG, Tween	IV	Breast cancer
Trans-Retinoic Acid	Atragen	DMPC, Soybean Oil	IV	Renal Cell Carcinoma
Irinotecan and Floxuridine	CPX-1	DSPC, DSPG, Chol	IV	Colorectal cancer

Liposomal API Solubilities

Aqueous Solubility of API's in Common Liposomal Formulations

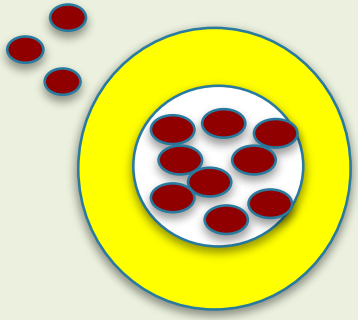
API	Solubility (mg/ml water)	
Amphotericin B	0.08*	(Water insoluble)
Daunorubicine	10	
Verteporfin	0.02	(Water insoluble)
Cytarabine	1000	
Morphine Sulfate	64	
Vincristine	2.27	
Irinotecan	25	
Cysplatin	0.8	

Most Liposomal drugs contain API's with good water solubility

*) Lowest literature value identified for Amphotericin B. Most state "water insoluble". Our experience suggests <1μg/ml

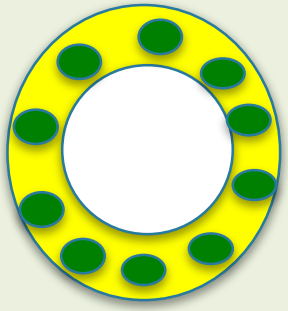
Major Differences in Liposomal Formulations

Doxorubicin ( )



- Doxorubicin is water-soluble (10 mg/ml)
- The liposome is merely a packaging and delivery tool; it encapsulates the drug in lumen
- API does not interact with the bilayer
- Drug parameters inside the liposome identical to aqueous solution

AmBisome ( )



- AmBisome is water-insoluble (<0.08µg/ml)
- The space enclosed in the lumen is irrelevant
- API interacts with the bilayer in a complex manner
- Drug status inside the bilayer is different than as free

Complexity of AmBisome may be highly underestimated without understanding these major differences

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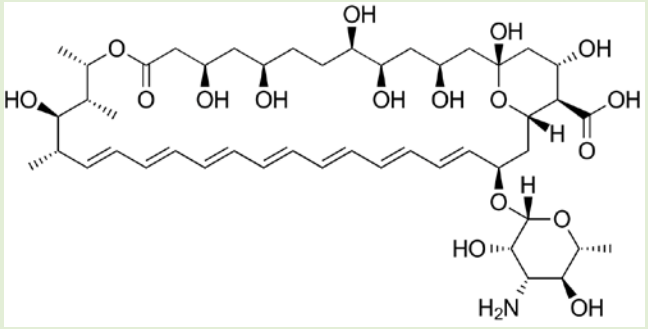
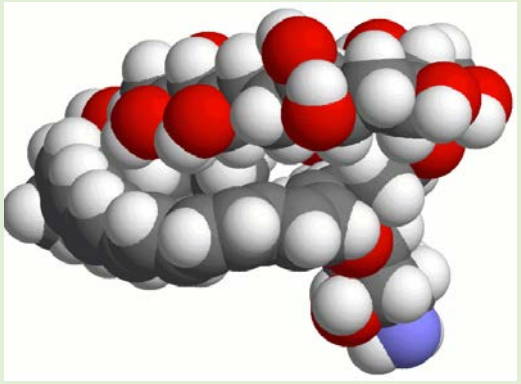
What is Amphotericin B?

- Chemical: A polyene antibiotic, MW 924 Da
- Source: The filamentous bacterium *Streptomyces nodosus*
- Medical Usage: Potentially life-threatening fungal infections, as well as anti-protozoa (e.g. leishmania, amoeba). Little acquired drug resistance. WHO list of essential medications
- Molecular structure:





- Physical properties:
  - Color: Yellow to orange
  - Solubility: Aqueous, <0.1 mg/ml (< 1mM) at neutral pH
  - Solubility: Methanol, 200 mg/ml

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History of Amphotericin B and Its Pharmaceutical Products

- **1955** First discovered from a bacterial strain in soil from the Orinoco River
- **1959** Fungizone formulation introduced to the market by Squibb, ("AmBD", 'Conventional' formulation, Deoxycholate Suspension)
- **1970** First X-ray structural elucidation
- **1987** First chemical synthesis by Nicolau
- **1995** FDA approves Abelcet (AmB in DMPC/DMPG lipid mix)
- **1997** FDA approves AmBisome (AmB in HSPC/DSPG/Cholesterol unilamellar liposomes)
- **2016** AmBisome patent (Nektar to Gilead) expires

Liposomal AmB Regulatory Guidelines (2016)

Contains Nonbinding Recommendations

Draft Guidance on Amphotericin B

This draft guidance, when finalized, will represent the current thinking of the Food and Drug Administration (FDA, or the Agency) on this topic. It does not establish any rights for any person and is not binding on FDA or the public. You can use an alternative approach if it satisfies the requirements of the applicable statutes and regulations. To discuss an alternative approach, contact the Office of Generic Drugs.

Active Ingredient: Amphotericin B

Dosage Form; Route: Liposome injection; intravenous

Recommended Studies: Two studies

When the test and reference liposome products

- have the same drug product composition and
- have equivalent liposome characteristics, including liposome composition, liposome size distribution, number of lamellar, electrical surface potential or charge, lipid bilayer phase transition, and in vitro leakage rates.

The following in vivo and in vitro studies are recommended to demonstrate bioequivalence:

In Vitro Study:

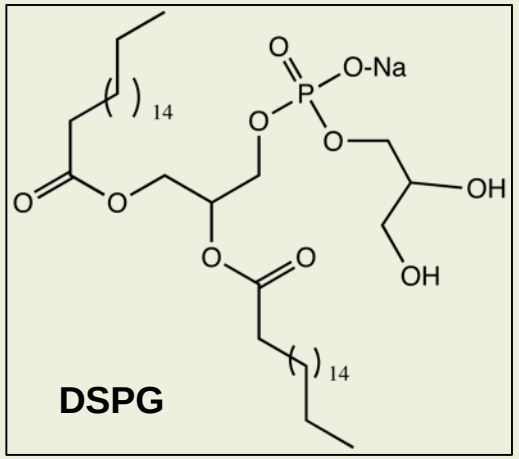
B. Type of study: Liposome size distribution
Design: In vitro bioequivalence study on at least three lots of both test and reference products

Equivalence based on (95% CI): Population bioequivalence based on D_{50} and SPAN [i.e. $(D_{90}-D_{10})/D_{50}$] or alternatively on the harmonic intensity-weighted average particle diameter and polydispersity index derived from cumulant analysis of the size intensity distribution.

Formulation and Process in the Innovators Patent

Drug Product Composition

Ingredient	AmB Patent Example 1	AmBisome Product Insert
API	+	+
HSPC	+	+
DSPG	+	+
Cholesterol	+	+
Succinate	+	+
Disaccharide	Lactose	Sucrose
Tocopherol	-	+



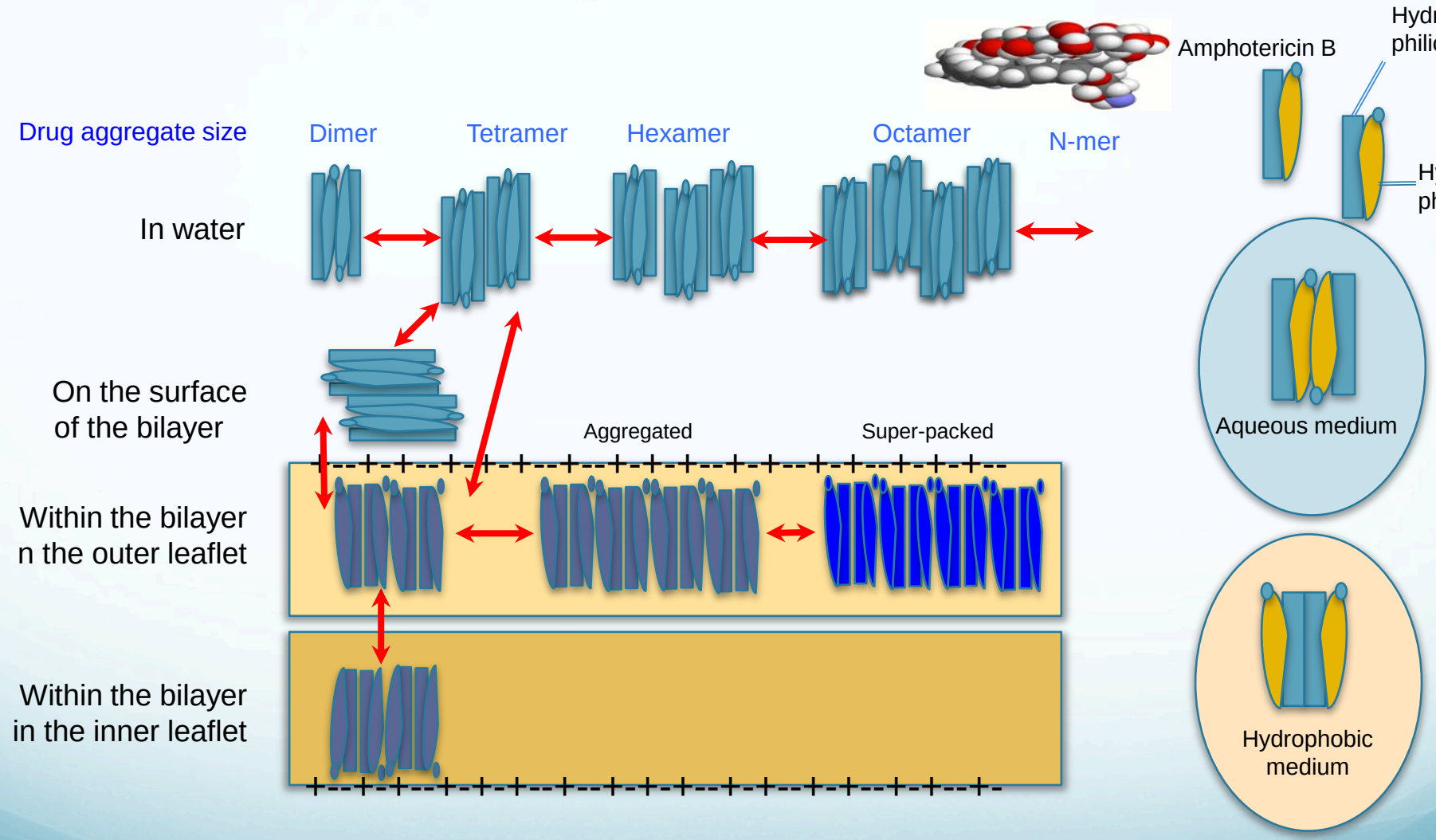
DSPG

Manufacturing Process

Step	Process	Purpose
I	Organic Solvent Mixing	API dissolution, Drug-lipid complex formation
II	Spray Drying	Removal of Organic Solvents (MeOH, $CHCl_3$)
III	Microfluidization	Lipid hydration & formation of SUV (liposomes)
IV	Lyophilization	Long term DP preservation in a dry state

Drug-Drug Interactions

Aggregation Dynamics and Packing in Different Media



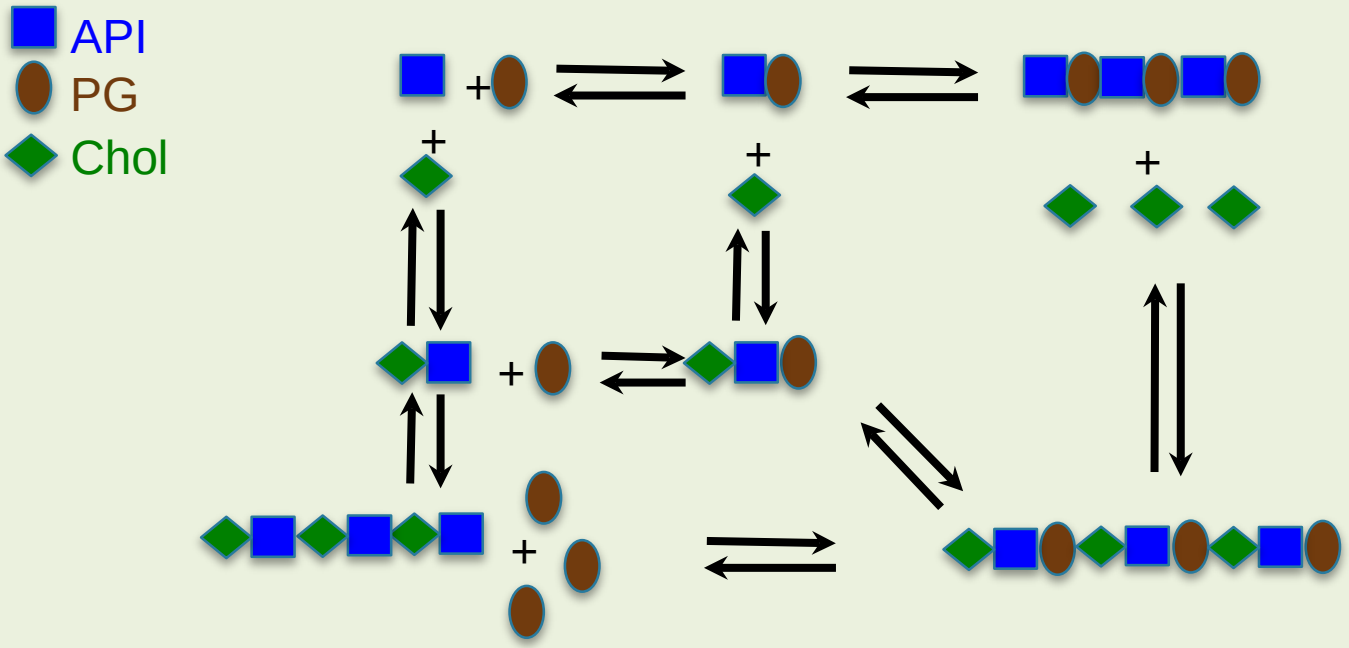
The free Amphotericin B is aggregated both in water and within the membrane

Drug configurations depend on a complex set of kinetic and thermodynamic relations that span multiple media and inerfaces

Must be considered when discussing the meaning of "incorporation", Free/bound issues, efficacy and toxicity.

Drug-Lipid Interactions
Additional Complexity in Aggregate Formation

Possible modes of interaction between API and key lipids



- PC provides the bilayer (hydrophobic) milieu, but plays less of a role in specific API aggregation and API-lipid interactions (PC is not protonated in the acidification step)
- API-Lipid interaction is critical but complex. Different types of aggregates may be formed
- Process Step I details may be critical for ultimate DP performance

Key Issues in Analytics and Process
Solubility and The Physical State of The API

Aggregate Size/Packing Variability

- Source of API (solid/solvent)
- Sonication
- Time in solution/ solutes
- Temperature
- Auxiliary solubilizing media
  - Detergents (CTAB, DOC in Fungizone)
  - Lipids (Abelcet)
  - Lipid bilayers (AmBisome)

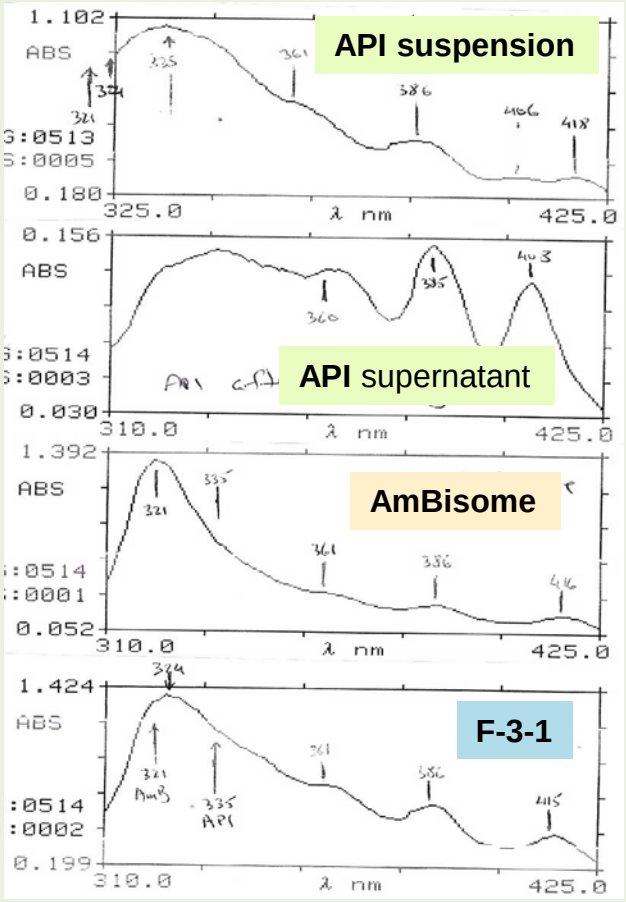
Implications

- Reproducibility
- Free-bound (?)
- API packaging in bilayer
  - Spectral features
  - Stability
  - Efficacy
  - Toxicity

Analytical Methods
Methods Used – Absorption Spectoscopy

UV/Vis Assessment of Aggregation

- Buffer: Saline Succinate, pH 5.5
- Concentration: API conc. adjusted to 15-20 μM (~14-18 μg/ml assessed in methanol)
- Absorption spectra measured at 310-425 nm



Quantitation of API Packing (AmBisome values in red)

1. Absorbance Peak for the super-aggregates, 321-322 nm
2. "Red Shift" of the main absorption signal is indicator of a "quality" of the packing (e.g. pure API shift is 10-30 nm, NAT preps 0-15 nm); AmBisome shift ="0"
3. Ratio between non-aggregate peak (415 nm) and the main peak (320-350 nm), OD_{321}/OD_{415} = 0.15-0.25

Analytical Methods
Methods Used - K^+ release from RBC

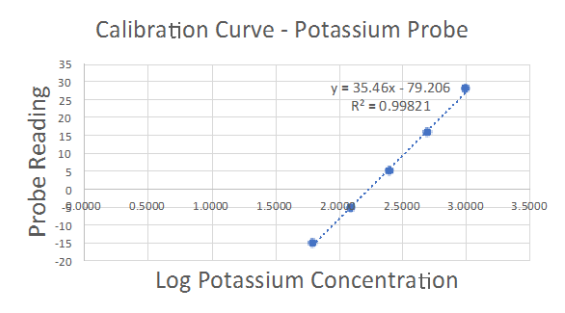
K^+ Release from RBC (In Vitro Toxicity Assay)

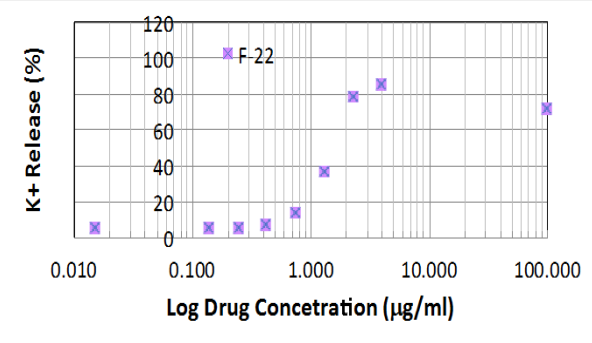
Principles

- Toxic API forms in the DP cause formation of pores in the RBC membrane, leading to leakage of univalent ions.
- Measure the released K^+ (using a potassium electrode) as in-vitro toxicity

Issues

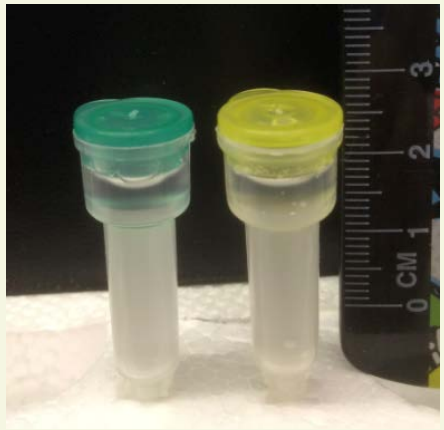
- The assay depends on sensitive biomaterials (Rat red blood cells)
- Variability in mV measured with age of electrode
- Run-to-run variability in AmBisome, low saturation





Free/Bound Assay
Spin Membrane and Spin Columns

- Spin membranes:
  - API not passing through 100 KDa MWCO filters, only passable if solubilized by detergent cetyl trimethylammonium bromide (CTAB)
  - Centrifugal force may be mandatory
  - Liposomes do not cross the membrane
  - API/Liposome mixtures cannot be separated using detergent
  - Conclusion: Method impractical for this purpose
- Spin Columns:
  - Good results with AmBisome (98-102% pass)
  - Good differentiation from API
  - Issues with reproducibility
  - May require a second analytical test for PL
  - Conclusion: Potentially useful method for Free/Bound



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Free/Bound API Assay
Why is it important?

- API is monomeric only at <1 microg/ml (produced only by water dilutions of DMSO solutions, not free API in water), heavily aggregated at higher concentration suitable for analyses
- API not exposed to water in the process HENCE: how do you do spiking??
- API is complexed with Cholesterol and PG in step I. Never in free form
- It is not clear what mediates the toxicity
  - Water born aggregates?
  - Liposome surface bound aggregates?
  - Membrane embedded small aggregates?
  - Larger membrane-embedded aggregates, not super-aggregates?
- The explored method (spin column) is likely to have ~5% in accuracy, Expect 5% “free” even if the outcome is 100% ‘encapsulated’ (note reservation on this term)

Manufacturing Process: Parameters Evaluated

Step I -API Solubilization and Complexation with Lipids*

- **Drug lipid complex formation**
  - **Acidification** - Found very important for API complexation with PG (slides below)
  - **API dissolution –** API at ~4mg/ml is 10-20-fold above solubility in methanol, and 10,000-fold above solubility in aqueous neutral buffers. Lipid complexation fills the solubility gap. Key questions: How much lipid? What lipids?
- **Chemistry and biophysics**
  - **Density -** Drug concentration relates to kinetics of complex formation with self, or with lipid molecules. (slide below)
  - **Charge** - Does net negative charge play a role in drug packing?
  - **Sterol –** Cholesterol mimics ergosterol, a membrane ligand target for Amphotericin B in fungal cell walls. May be a component in pore formation.

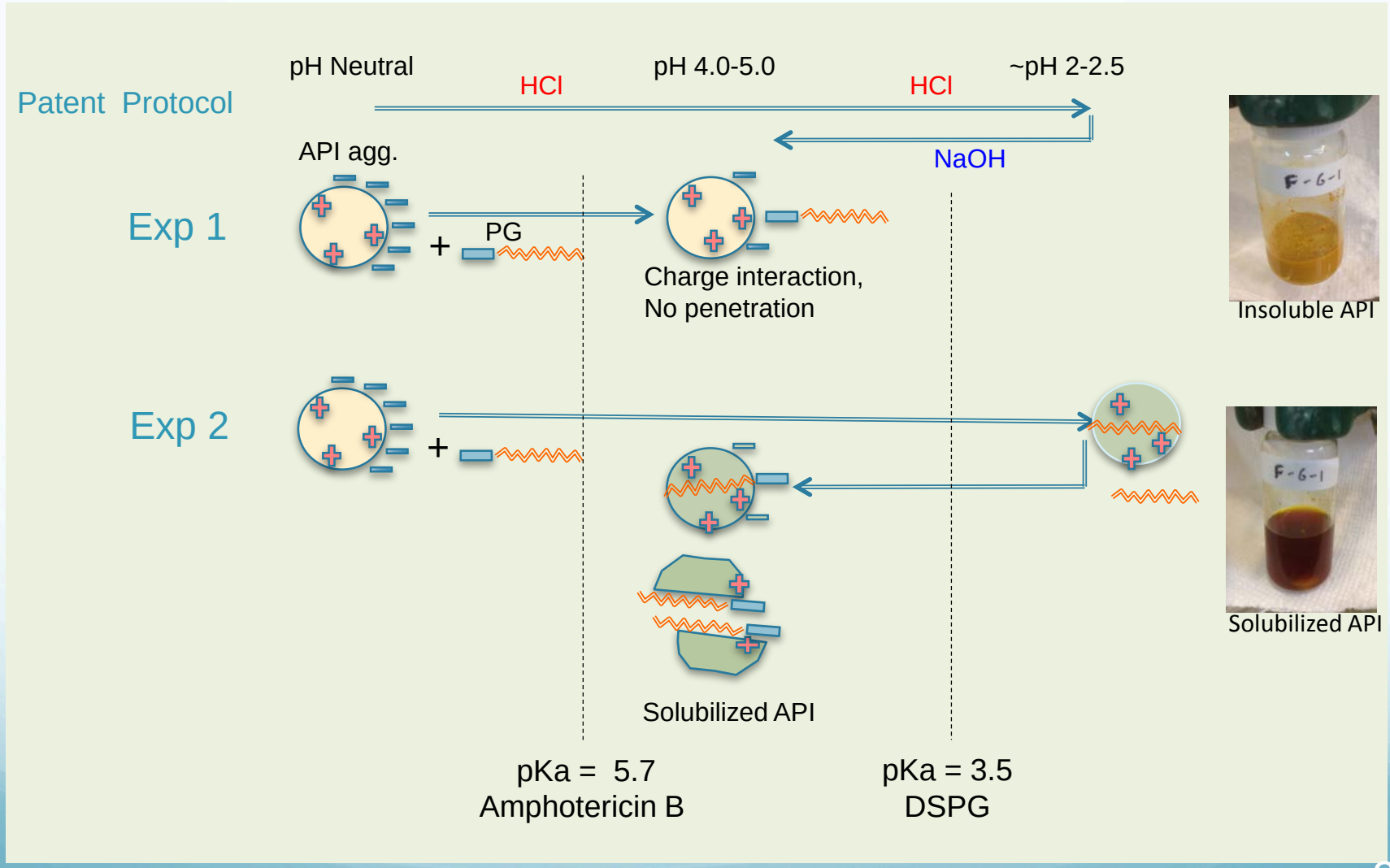
Manufacturing Process: Parameters Evaluated

Step I –Design of the QBD space

- **QbD approach**
  - The process includes more parameter than those tested. Limited by time!
  - Variations in this study ranged up to 15-40% (around the patent values)
  - Wanted to gain understanding. Generic approval process allows 5%. If 20% deviation works, 5% will certainly be included in the established QbD design space.
- **Process Parameters (CPP) Addressed in the QbD Phase:**
  - PG:Total PL ratio (molar)
  - API:Total PL ratio (molar)
  - Cholesterol:Total PL ratio (molar)
- **QbD challenges in Step 1**
  - No immediate analytical means to verify good/bad outcomes of the Step I
  - Attributes for all Step I CPP evaluated only after completion of the Step III

Manufacturing Process -Step I

Role of Charge Neutralization by Acidification



Manufacturing Process – Step I
pH/HCl Acidification - Summary

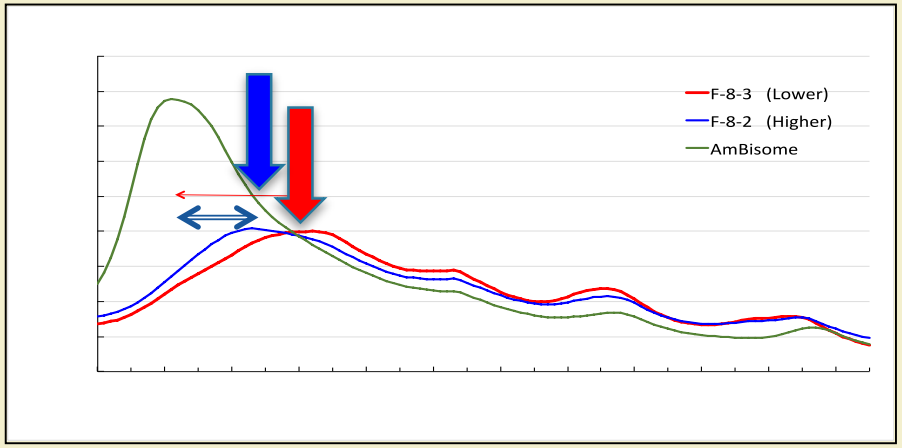
- Necessary for dissolution (see pictures)
- Issues with pH testing
  - Non aqueous. Water 1.7%, no phase separation
  - Impossible to sample hot solvents
  - Need dilution to measure pH (values erroneously high)
  - Litmus paper subjective, need wetting

API not dissolved

API Dissolved

- Resort to molar ratios
  - Patent HCl/API 1.84 HCl/PG= 0.95 (API/PG=0.52)
  - Failed 40% over acidification 2.21 1.14
  - Successful 20% under acid 1.20 0.62
  - Lower limit: one equivalent to API. Upper limit: one equivalent to PG
- API Degradation by excess acidification
  - Demonstrated by HPLC
  - Demonstrated by UV/Vis spectra (previous slides)

Manufacturing Process – Step I
API/PL Molar Ratio



Conclusion: Higher drug load improves packaging in bilayer. Confirmed in QbD screening

Specimen	Main Peak	Red Shift (nm)	416/Main
AmB	321	'0'	0.229
F-8-2 (+18%*)	333	12	0.38
F-8-3 (-20%*)	340	19	0.39

*) Percent changes on API/PL ratio, relative to the patent values

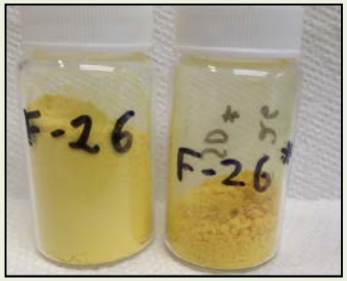
Manufacturing Process - Step II

Solvent Removal by Spray Drying

- Equipment Selection:
  - Dimensions, distances, volumes and velocities vary amongst SD instruments
  - Optimal conditions to be established for each SD model
- Parameters addressed in QbD:
  - Inlet temperature
  - Aspirator Velocity
  - Specimen feed rate
  - N_2 Flow rate
  - Specimen Temp.

Interplay of parameters determines the rate of drying, powder consistency, residual solvent. Higher T cause smearing on the collecting cylinder walls

Quality of the injected mist



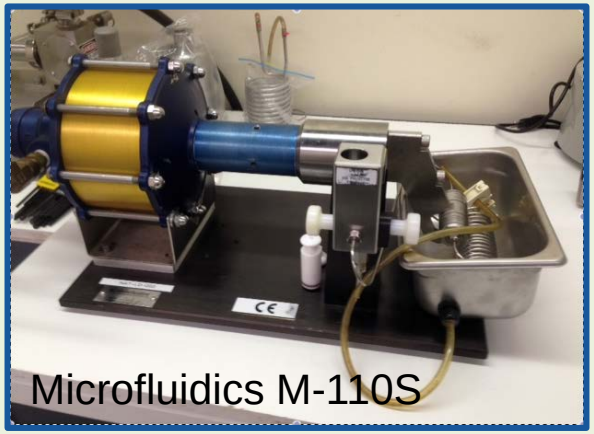
- Outcome*:
  - Powder recovered in the collecting jar is finer and lighter, material scraped is darker and more flaky
  - Powder hydrates better, no difference in final product performance*

*) Product attributes for all Step-II CPP's were only evaluated on the liposomal product following Step III of the process. Performance was evaluated primarily in the QbD work

Manufacturing Process – Step III

Lipid hydration and Microfluidization

- Equipment Selection:
  - Like spray drying, microfluidization parameters may vary among instruments. Re-evaluate on other equipment
- Specimen Temperature:
  - Lipids may shear differently when in the liquid crystal or gel phases.
  - Temperature may be important at the shear process.
  - Difficult to control in lab scale apparatus
- How intense a shear is required? (slide below)
- QbD parameters evaluated
  - Specimen temperature (^{o}C)
  - Pressure applied (psi)
  - No. of passages through the shearing cell



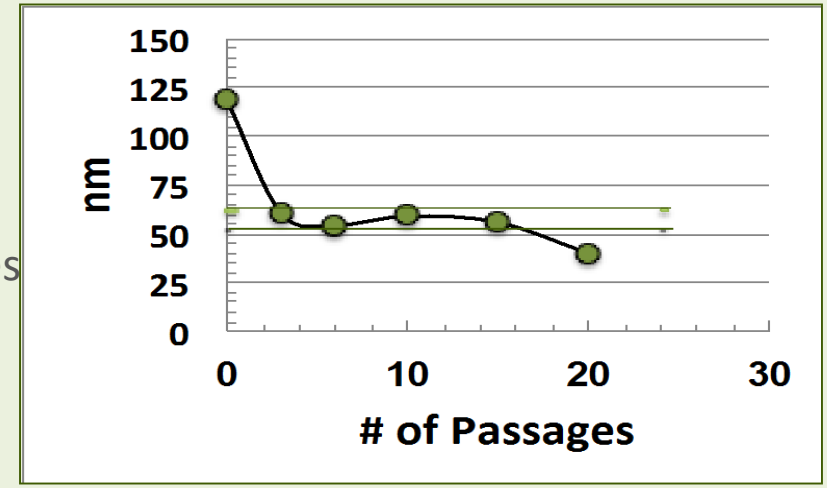
Microfluidics M-110S

*) Liposomes are already formed upon hydration as large Multi-lamellar Vesicles (MLV). The final DP, following microfluidization consists of Small Unilamellar Vesicles (SUV), namely single shell spheres.
**) Performance was evaluated primarily in the QbD work.

Manufacturing Process -Step III

How Many MF Passages are Required?

- Dry Lipid/API mixtures were hydrated with LS buffer (9 % lactose in 10 mM succinate buffer, pH 5.5)
- Specimen was probe sonicated for 45 s to break clumps
- Suspension was microfluidized
  - Specimen T= 55°C
  - Pressure 100 psi
  - Graph of particle size vs. no. of passes

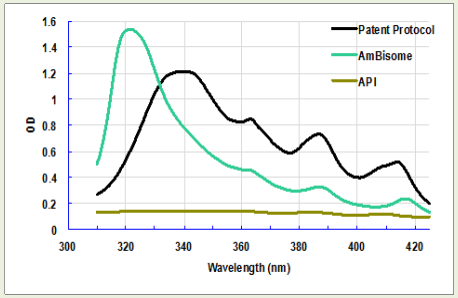


- Conclusions: Stable particle size similar to AmBisome (<100 nm) requires a small number of passages (QbD screening work tested - 4, 7, 10 pass.)

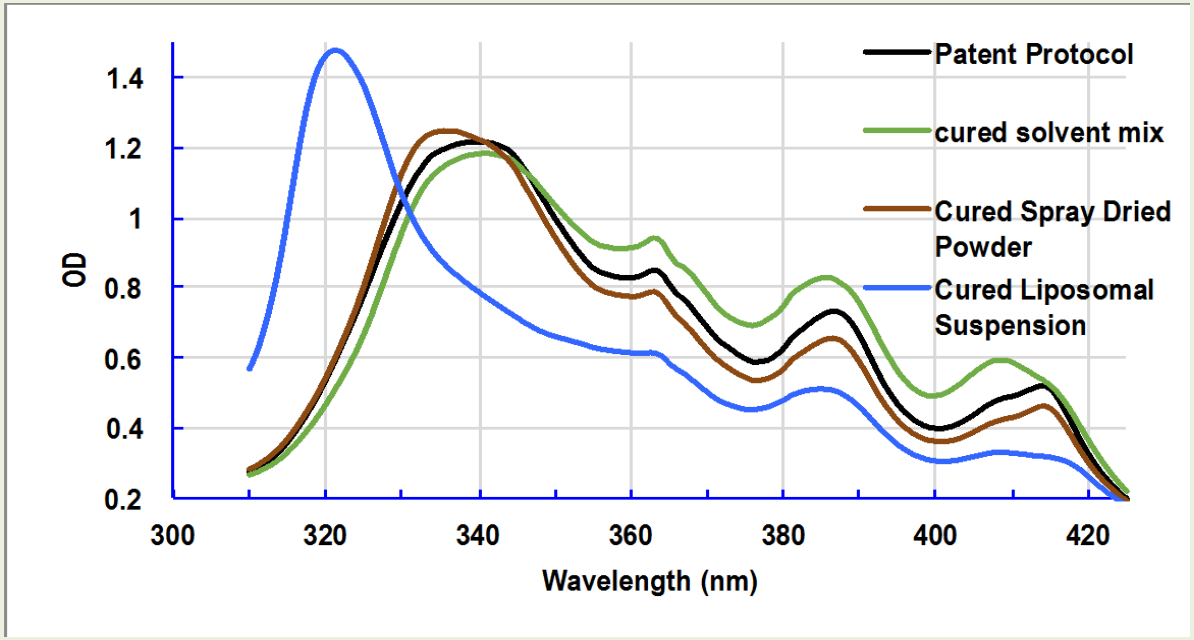
Manufacturing Process - Step III

UV Signature of Superpacking

Results:



Step	Peak	Peak Ratio
I	341	0.501
II	336	0.370
III	321	0.223
AmB	321	0.152



Conclusions:

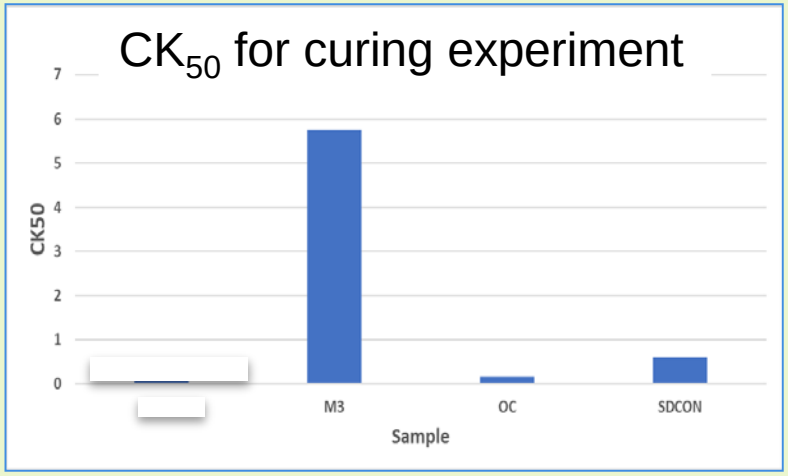
- Curing at prolonged periods is required for "super-aggregation" of API in the DP
- API reorganization happens within the bilayer, NOT detected in Steps I, II

Manufacturing Process -Step III

'Superpacking' Reduces Drug Toxicity

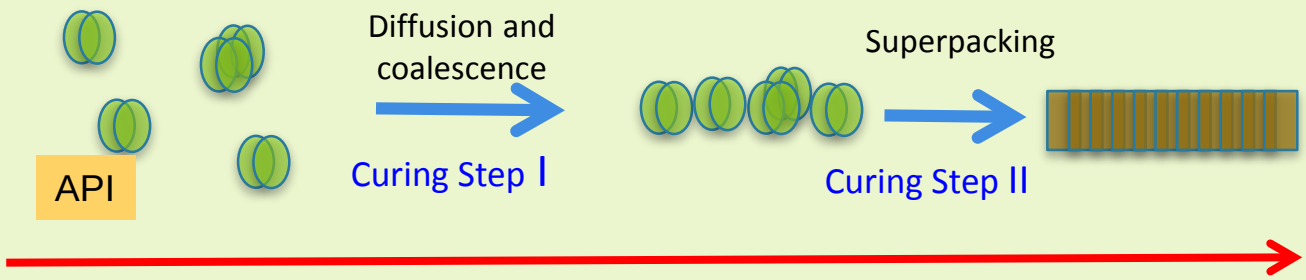
K^+ release from rats RBC
In vitro assay

'Curing' Process Step	CK_{50} (µg/mL)
AmB	**(4-20)**
Solvent Mix (I)	**0.325**
Spray Dry (II)	**1.191**
Microfluidization (III)	**11.508**



Conclusion: "Curing" the API after lipid hydration REDUCES toxicity by one order of magnitude in concentration

Manufacturing Process - Step III
Two Step Model of 'Superpacking'



What is super packing?

Much is yet to be learned on this process:

Some possibilities may include one or more of the following

- A conformational change of monomers within the aggregate?
- An aggregation sequence that need to reach a certain size?
- A reorganization aligning the API charges with the charges at membrane stern layer
- A formation of circular or other geometries out of other initial geometries?
- A squeezing out of PG or Cholesterol or both form the loose aggregate?

'Super-packing' of aggregated API is an intra-aggregate re-configuration

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