2012

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Degree Type

Open Access Senior Honors Thesis

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Lipid Membrane Disruption by Amylin in Type II Diabetes Mellitus: Effect of Head Group Loss

By

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A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Chemistry

Approved at Ypsilanti, Michigan, on this date April 12, 2012

Lipid Membrane Disruption by Amylin in Type II Diabetes Mellitus: Effect of Head Group Loss

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12 April 2012

Abstract:

Damage to insulin-producing beta cells of the pancreas in individuals with Type II Diabetes Mellitus is a traditional effect of the disease, which is often aggravated by age. A potential explanation for beta cell damage is disruption of the cellular lipid membrane from amylin accumulation. This presentation details an examination of possible age-related effects to membrane lipids, specifically differing levels of head group loss, through treatment of membrane models with amylin and dye leakage assays.

Introduction/Background:

In America and many other parts of the world today, one of the most common autoimmune disorders is Diabetes Mellitus, with more than 1 in 10 Americans suffering from some form of the disease[1] Characterized by deficient mechanisms for insulin production/absorption by the body, untreated diabetes can quickly lead to detrimental side effects such as ketoacidosis or neuropathy. In addition, many diabetic patients (especially Type II diabetics, which account for 90% of all Diabetes cases) are diagnosed with the disease when they are already out of shape or eating a poor diet, which increases the risks for heart conditions and other illnesses. [2,3] Diabetes has also been shown to affect the elderly, with 1 in 4 Americans over the age of 65 diagnosed with some form of Diabetes. [1] This statistic, along with increased insulin resistance and insulin-producing β-cell damage in older diabetic patients, is cause for concern.

Insulin is a hormone vital to the digestion and absorption of glucose into all cells for energy. In the human body, the β-cells of the pancreas are responsible for the production and dispersion of insulin throughout the body. In Type II diabetic patients, the gradual loss of β-cell function is one of the major factors in determining the extent of complications such as hyperglycemia. As patients age, a clear trend towards decreased β-cell function as well as increased insulin resistance is often shown. [4] What characteristics of aging cells cause these issues to propagate? One potential answer lies within the cellular membrane of the β-cells themselves. If the β-cells change the chemical makeup of their lipid membrane due to the ageing process, it is possible that the susceptibility of the membrane to damage and degradation would also increase. This project set out to examine the effect of Human Islet Amyloid Polypeptide(HIAPP), more commonly referred to as amylin, on various types of lipid membranes.

Besides the production of insulin, other hormones that serve a similar function have also been shown to be produced in the pancreatic β-cells. One of these hormones, amylin, is responsible for not only controlling blood sugar in a similar method to insulin, but also promoting weight loss by slowing the rate at which the body empties the digestive system. [3] Amylin is secreted in a roughly 1/10 ratio with insulin [6], and has a 37-amino acid residue structure (shown in Fig 1) that has a net positive charge of +3 at physiological pH, due to the free N-terminal, K1 residue, and R11 residue.[7,2,8,9] Amylin has been shown to induce a toxic effect on the lipid membranes of insulin producing β-cells, and this is due primarily to the formation of fibril structures from amylin units. [5]

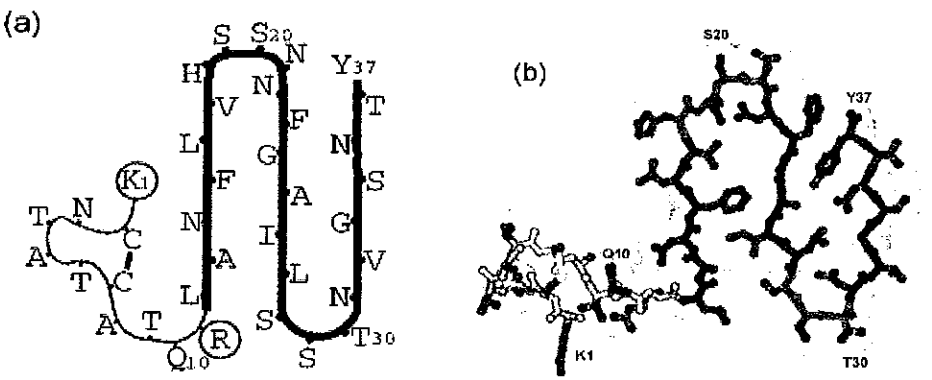


Fig 1: Chemical structure of amylin in 2-D and 3D models. Charged residues in (a) are circled.

[8]

The degree with which amylin interacts with pancreatic cells is primarily based on charged interactions between the positively charged peptide and negatively charged membrane lipids. [9] While the lipid membrane of a human cell is far more complicated than a simple lipid bilayer, the lipid composition is the most important factor in this investigation. The model membranes prepared in this study are shown below in Fig 2 - Fig 4. DOPC is a zwitterionic lipid containing a choline head group and is neutrally charged; DOPS is a similar lipid with a serine head group, causing it to be negatively charged; DOPA lacks any head group whatsoever and is also negatively charged. It is important to note that a normal pancreatic cell contains a ratio of roughly 30% negatively charged membrane lipids to 70% neutrally charged membrane lipids. In this study, this is represented by a control mixture of 7:3 DOPC and DOPS.

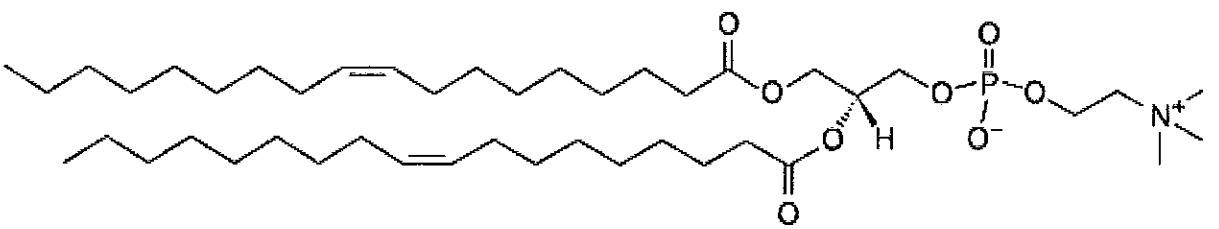


Fig 2: Chemical Structure of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC)

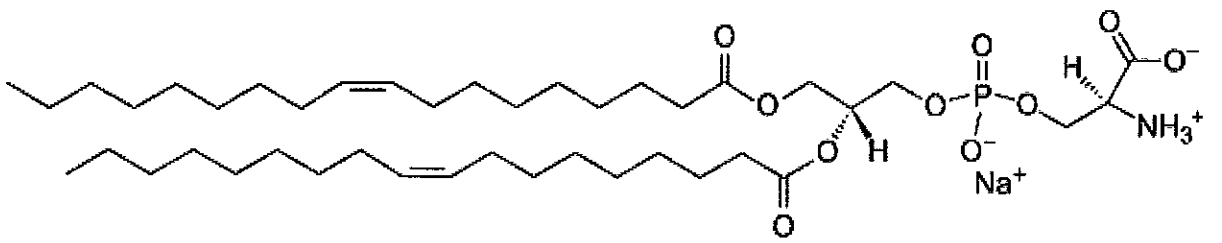


Fig 3: Chemical Structure of 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS)

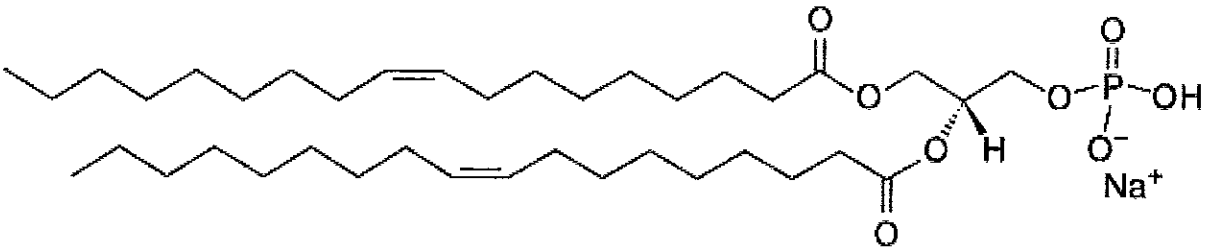


Fig 4: Chemical Structure of 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA)

The specific mechanism of amyloid fibril formation is detailed below in Figure 5. Individual amylin molecules quickly begin to form oligomers, followed by larger fibril formations. These larger structures interact with the lipid membrane due to the charged interactions discussed earlier. Pores, more specifically, charged ion channels begin to form in the membrane, eventually leading to the complete and total breakdown of the membrane itself and cellular death. Data from Mirzabekov et al. (1995)[7] has show this damage to be irreversible, with even a small exposure having potentially deleterious effects on membrane stability. Fibrils can form as aggregation increases, damaging the membrane even further.

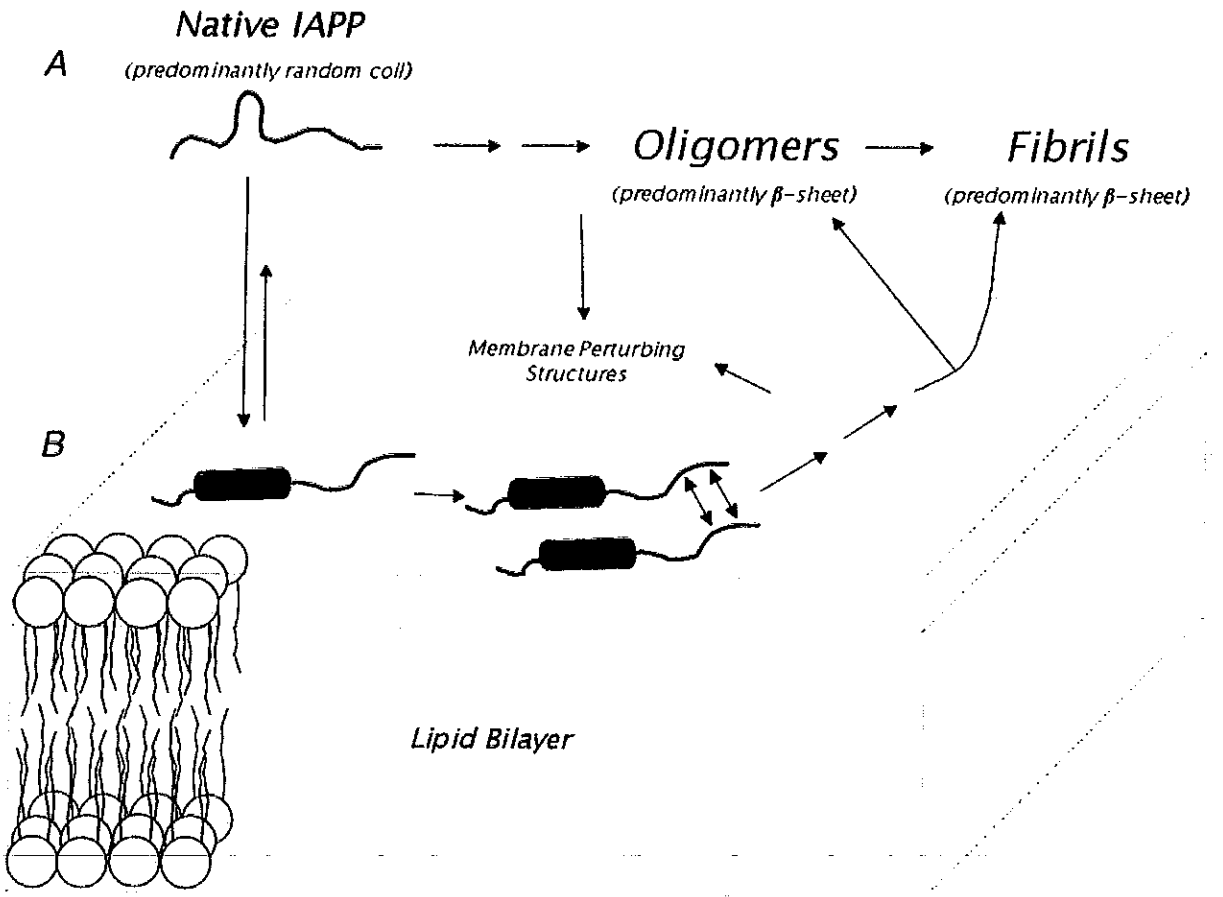


Fig 5: Diagram of Amylin (IAPP) showing oligomer formation and amyloid interaction with membrane [9]

As humans age, the membrane lipid composition of the β-cells within the pancreas show noticeable changes due to the process of ageing. [2,3,4] Diet also has a major effect on both the lipid concentration and the cholesterol content of the bilayer. [2,3,4,10] The combination of age and poor diet in many Type II Diabetes patients will likely lead to some adjustment of the membrane. Previous research has shown significant modification of the lipid structure in elderly patients. [4,10]We believe that lipids containing negatively charged head groups such as phosphatidylserine(PS) and especially those that are composed of lipids that lack a head group entirely such as phosphatidic acid (PA) will increase the likelihood of the

membrane interacting with the positively charged amylin, leading to pore formation in the lipid membrane and cellular death. In order to test this hypothesis, artificial vesicles containing carboxyflouroescein dye were prepared, each containing varying ratios of the three lipids detailed above in Fig 2 - Fig 4. By measuring the effect of amylin on the vesicles, specifically the amount of membrane degradation, it should be clear whether or not amylin has a more degenerative effect on membranes that have lost a head group and/or become more negatively charged.

Materials:

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), Avanti Polar Lipids

1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS), Avanti Polar Lipids

1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), Avanti Polar Lipids

Carboxyfluorescein Dye, Sigma Aldrich

Extrusion set with differing sizes of polycarbonate membrane (0.2-1 micron), Avanti Polar Lipids

G-50 gel beads, Sigma Aldrich

Amylin, Anaspec

Microplate Fluorescence Reader, Bio-Tek

Sodium Phosphate Buffer pH 7.5

Methodology:

The formation of artificial vesicles containing carboxyfluorescein dye was performed via a freeze-thaw process as follows. Each preparation contained 30 μM of carboxyfluorescein (~5.64 mg dissolved in 0.5 mL of a pH 7.5 Sodium Phosphate buffer), and was combined with a lipid preparation totaling 5mg+ 1mL of chloroform. The differing lipid preparations are detailed in Table 1. A control sample, simulating normal lipid composition, was prepared using a 7:3 ratio of neutrally charged DOPC: negatively charged DOPS. (3.5 mg DOPC: 1.5 mg DOPS and is not listed in Table 1)

Table 1: Lipid composition of assays. Samples 1 through 3 simulate increasing head group loss from negatively charged DOPS, while Samples 4 though 6 measure increasing head group loss from neutral DOPC

Assay number	Amt. of DOPC in membrane (mg)	Amt. of DOPS in membrane (mg)	Amt. of DOPA in membrane (mg)	Ratio of DOPC/DOPS/DOPA
1	3.5	1.0	.5	7/2/1
2	3.5	.5	1.0	7/1/2
3	3.5	0	1.5	7/0/3
4	3.0	1.5	.5	6/3/1
5	2.5	1.5	1.0	5/3/2
6	2.0	1.5	1.5	4/3/3

Freezing and thawing was undertaken using a dry-ice/acetone bath and warm water. Each set of samples was put through a cycle of 5 freezes and 5 thaws and then placed into a freezer until they were needed for the fluorescence assay. This procedure caused the formation of initial vesicles around the carboxyfluorescein dye.

When performing fluorescence assays using the vesicle samples obtained earlier, the following methodology was used. A tube of lipid was removed from the freezer, allowed to thaw, then collected in a gas tight 1000µL syringe and passed through a clean Avanti Polar Lipids Mini-Extruder with a 1 micron polycarbonate membrane. After several passes the sample was collected and the extruder disassembled, then re-assembled with a 0.2micron polycarbonate membrane in place of the original membrane. The sample was then carefully passed through the extruder exactly 21 times. This allows for vesicles of the desired size to be formed. The extruder was set aside and the lipid sample placed into a test tube while a gel exclusion column was prepared using G-50 gel beads and pH 7.5 sodium phosphate buffer.

Following the column preparation, the lipid sample was loaded onto the column, and run using the sodium phosphate buffer. Due to the exclusion limit of the gel inside the column and the fact that the vesicles which were created were significantly larger than any free dye particles, the vesicles will pass quickly through the column, leaving any dye behind to be slowed within the gel pores. The first yellow-colored fraction was collected from the column and set aside while the column was flushed with buffer and a 96 well microplate was prepared for the fluorescence assay.

Each fluorescence assay was prepared in an identical fashion, using varying concentrations of amylin to induce the leakage of the dye-containing vesicles. Eight samples were prepared in test tubes, and then divided into three identical microplate well samples of ~300μL. Samples were mixed by adding buffer into the test tube first, followed by amylin or DMSO, and finally adding 20μL of vesicle solution buffer (collected from the gel column). Each sample consisted of 1500 total μL, including all elements. The control sample contained only buffer and 20μL of vesicles. An additional sample was prepared to completely break open the liposomes using Triton-X detergent. This allows a comparison between the most concentrated amylin sample and the 100% leakage detergent sample. It is important to note that if the veracity of the liposomes prepared is at all in question, the 100% leakage sample and control sample should be prepared and examined before the other samples are prepared. This prevents the waste of amylin on vesicles which were not prepared properly. Sample preparation is detailed below in Table 2. Each sample was mixed thoroughly and set aside for the final fluorescence assay.

Table 2: Assay preparation for Fluorescence Assay

Trial	Control	100%leakage	1	2	3	4	5	6
Amylin (μL)	0	0	1	2	5	11	21	42
Vesicles(μL)	20	20	20	20	20	20	20	20
Buffer (μL)	1480	1390	1479	1478	1475	1469	1458	1438

DMSO (μL)	0	50	0	0	0	0	0	0
Detergent(μL)	0	40	0	0	0	0	0	0

For the fluorescence assay, each of the eight samples was divided into 300 μL fractions and placed onto a well plate. This provides triplicates of each sample and reduces the error in each sample. The well plates were placed into a Bio-Tek Flx 800 Microplate Fluorescence Reader for 3 hours with a reading interval of one minute. A sample of the raw data and calculations obtained from the fluorescence assays is detailed in supplemental table 1. This specifically refers to the control sample.

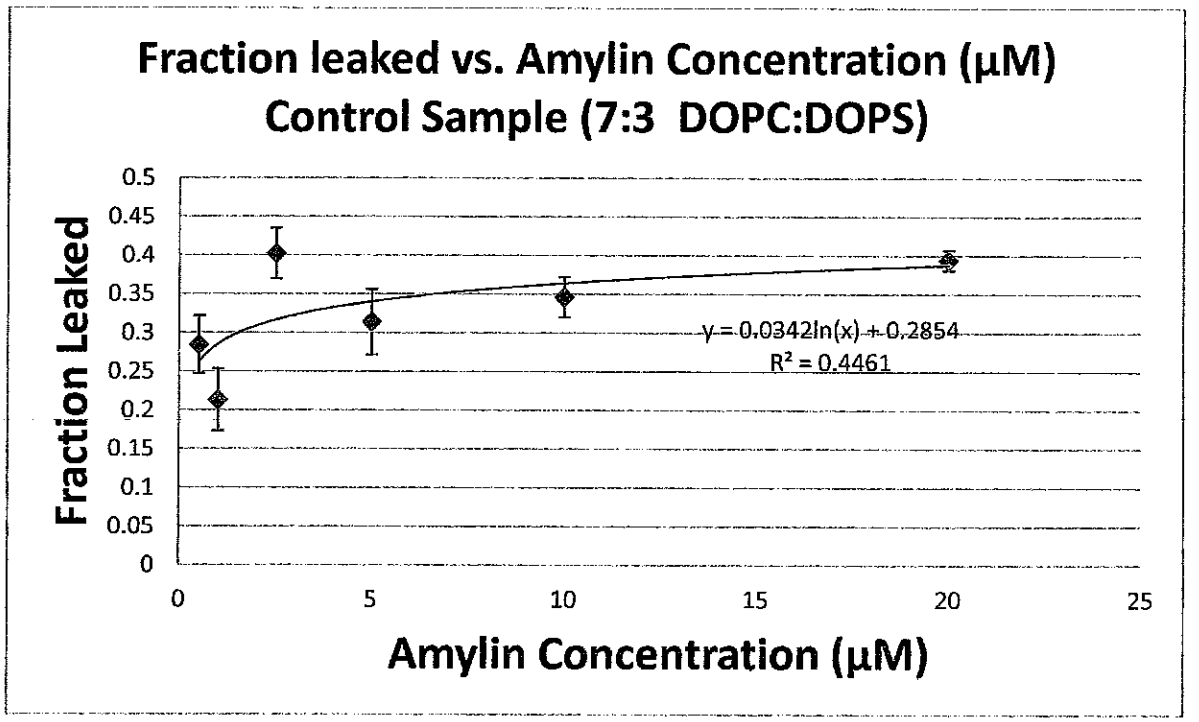
Results

In order to convert the raw fluorescence measurements into fraction of the dye leaked, several calculations were performed. The time-averaged fluorescence values from each set of three trials were combined and the average value from that combination was obtained. This value was placed into equation 1:

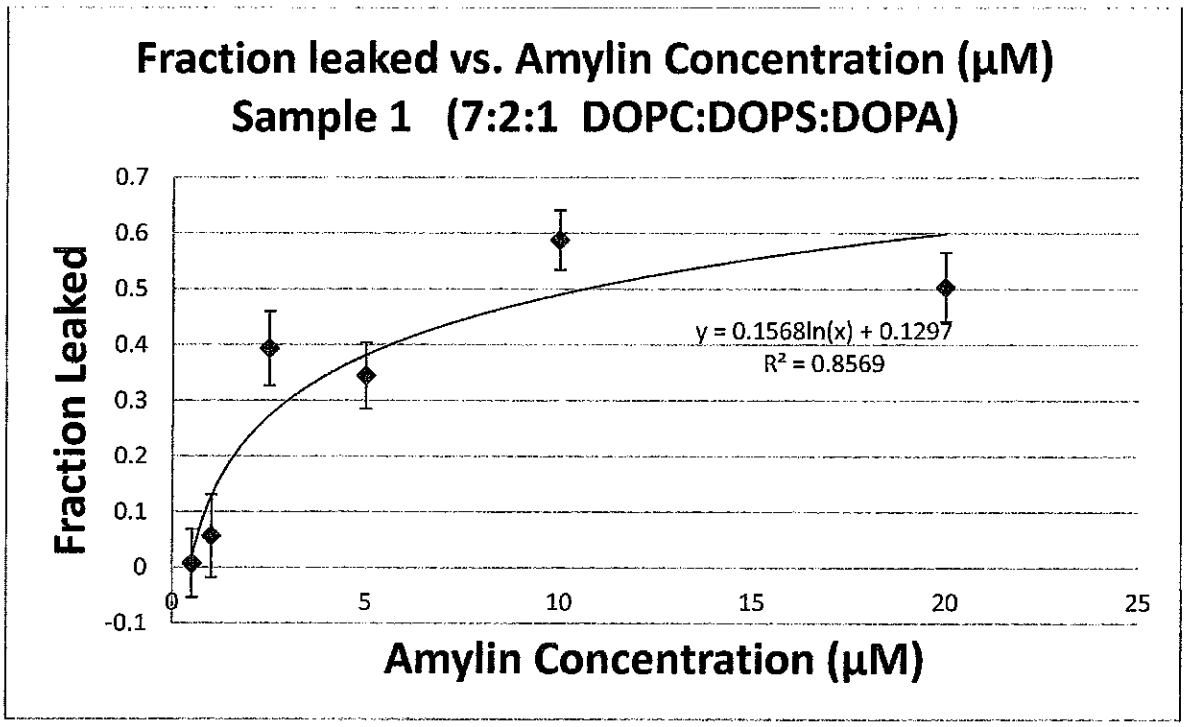
$$Equation\ 1:\ Fraction\ Leakage = \frac{Average\ from\ All\ Runs\ (Sample\ being\ examined) - Average\ from\ All\ Runs(Control\ sample)}{Average\ from\ All\ Runs\ (100\%\ Leakage\ sample) - Average\ from\ All\ Runs\ (Control\ Sample)}$$

These values were then interpreted graphically based on the concentration of amylin present in each trial, shown in Graphs 1 through 7. A logarithmic trend line was added to help

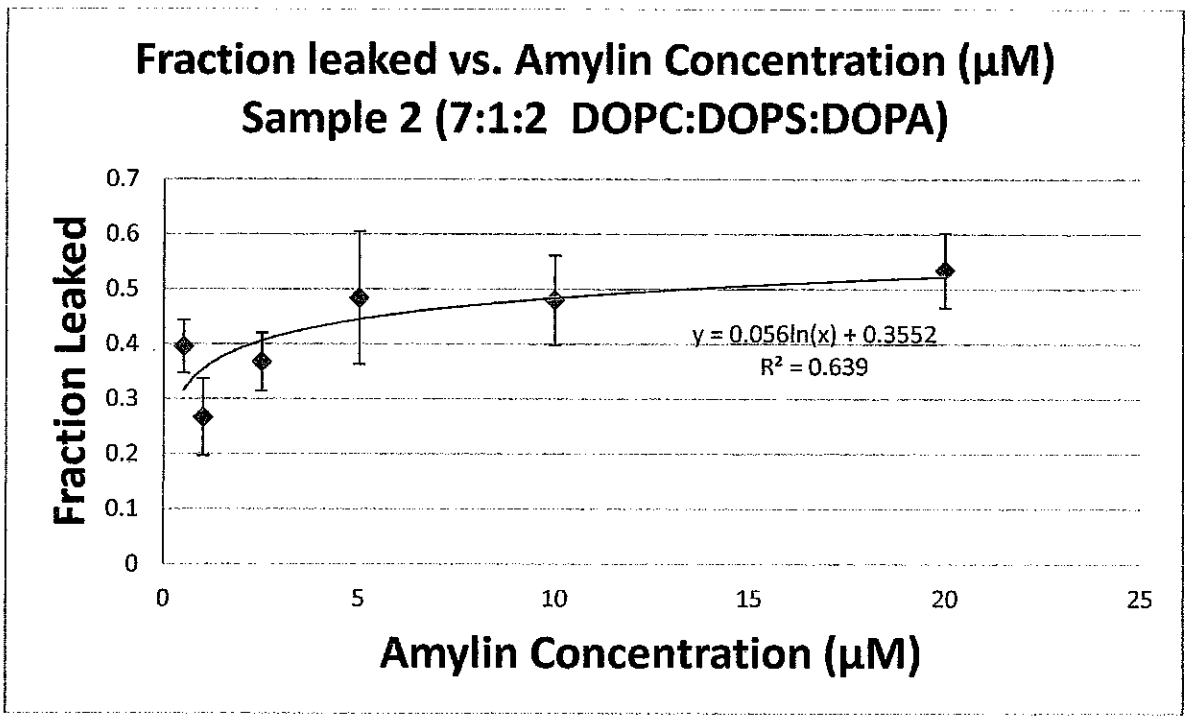
characterize the amount of leakage shown as the concentration of amylin was increased. While the correlation (R^2) values are lower that normally acceptable in some of the lower concentration tests, at the higher levels the data clearly show much higher levels of correlation, all above 0.90.



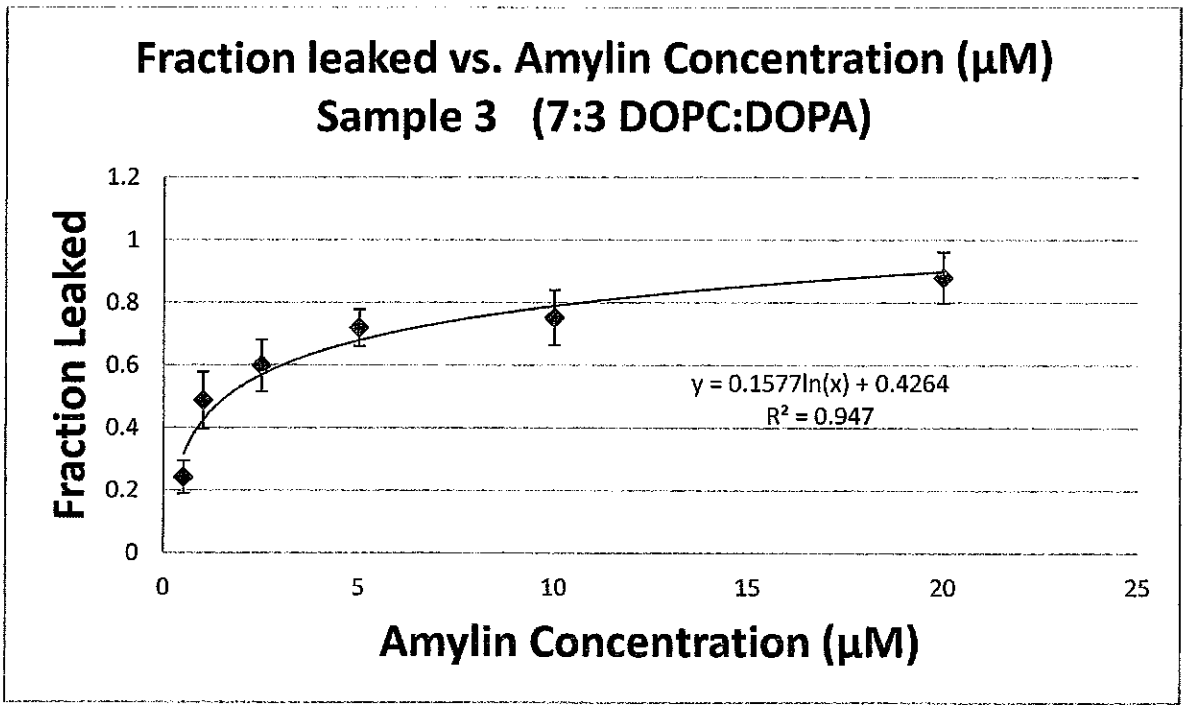
Graph 1: Control sample fraction leaked vs. amylin concentration



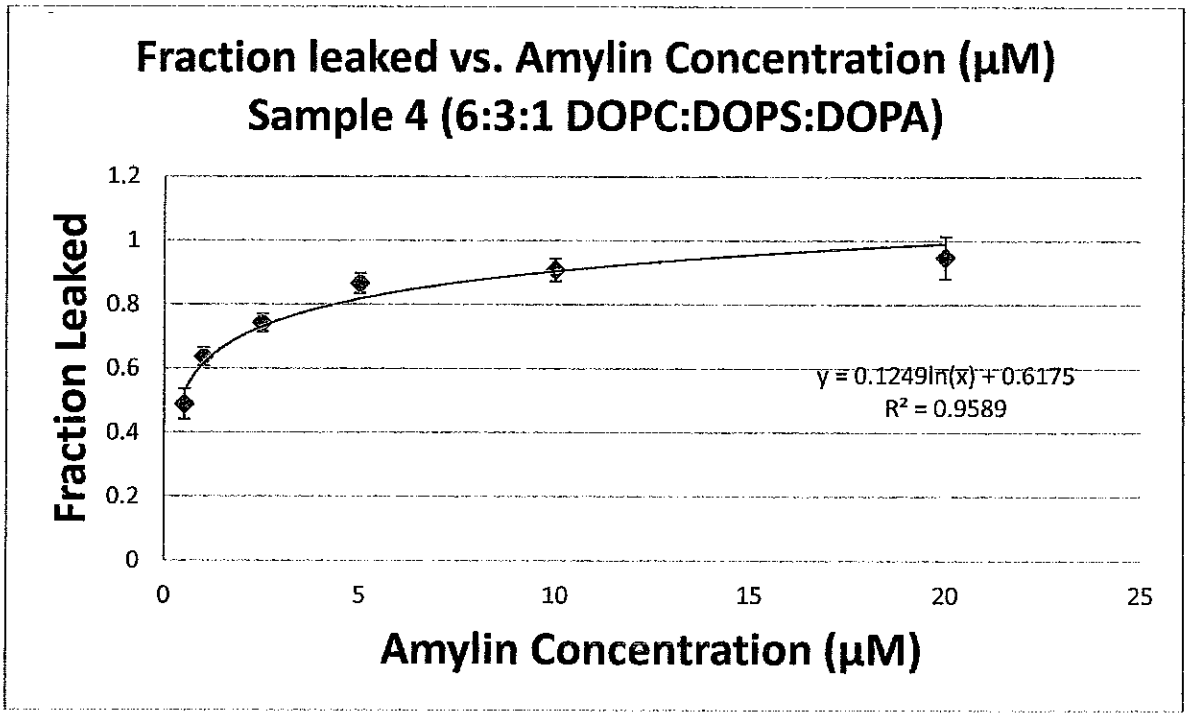
Graph 2: Sample 1 fraction leaked vs. amylin concentration



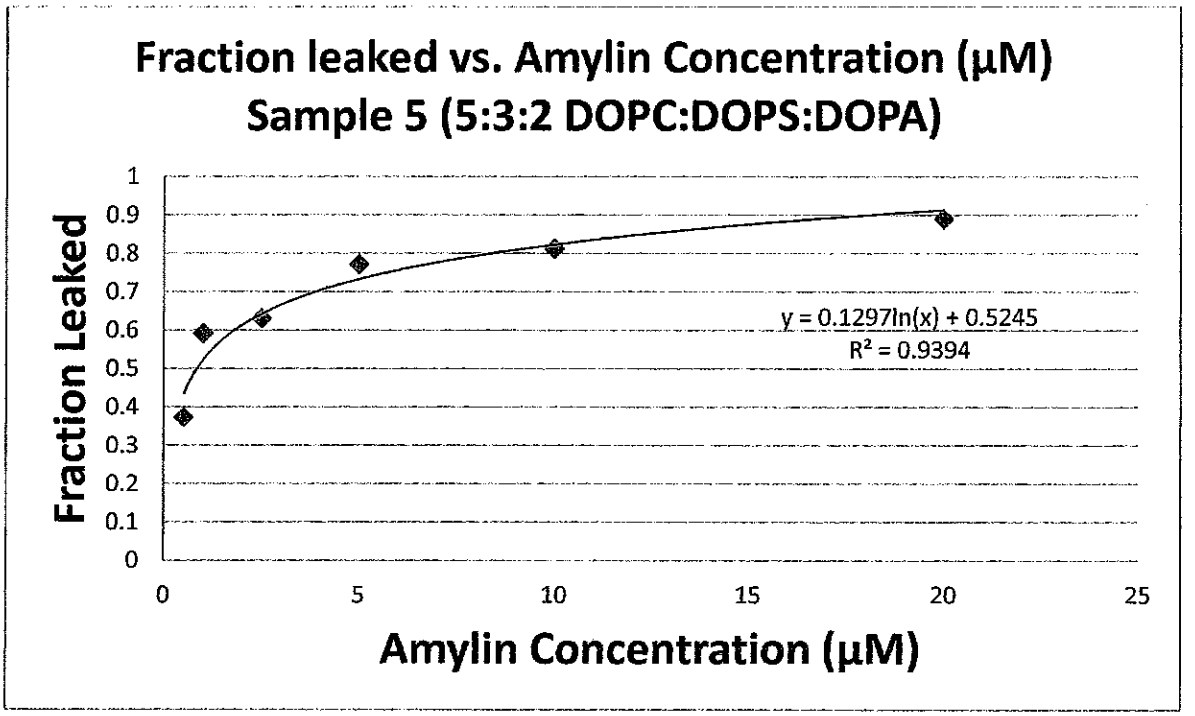
Graph 3: Sample 2 fraction leaked vs. amylin concentration



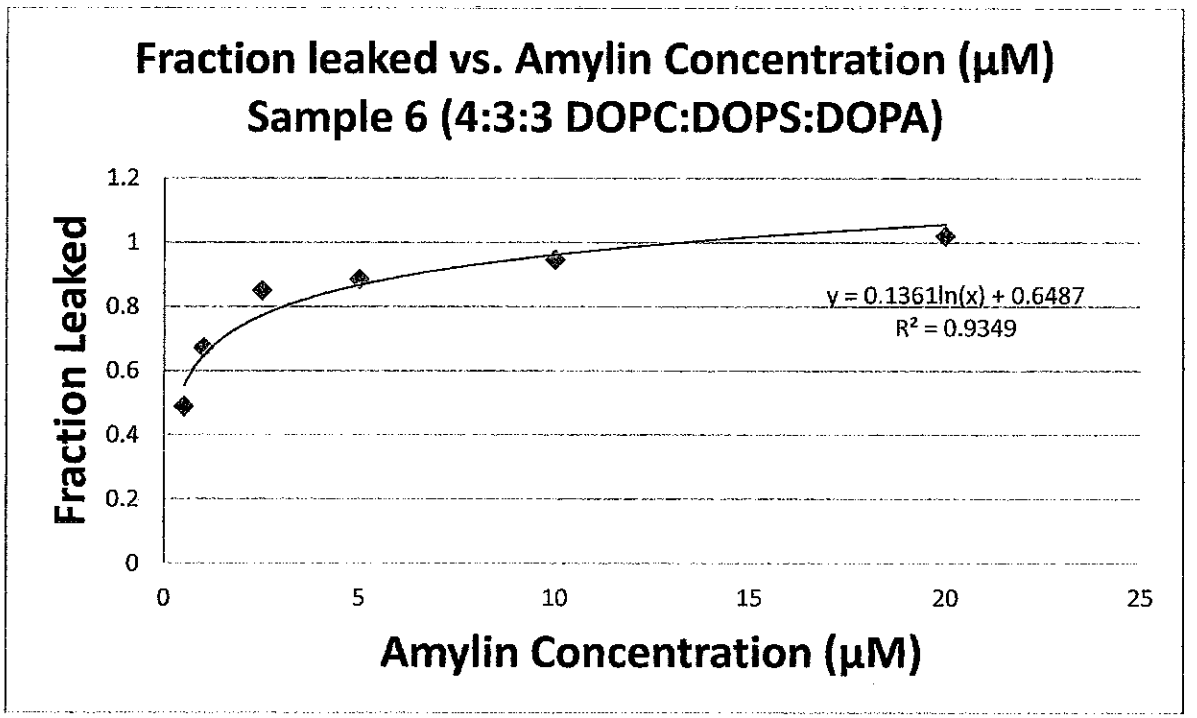
Graph 4: Sample 3 fraction leaked vs. amylin concentration



Graph 5: Sample 4 fraction leaked vs. amylin concentration



Graph 6: Sample 5 fraction leaked vs. amylin concentration



Graph 1-7: Sample 6 fraction leaked vs. amylin Concentration

The data clearly show a progression of increased leakage as the head groups of the lipid membranes are removed, simulating the loss of head groups due to age. Even at the lowest concentration of amylin, the amount of leakage in all samples was as high or higher than the highest leakage levels in the controls. If amylin was not responsible for the degradation of the membrane, this increased leakage would not be observed. Each sample exhibits a rough level of concentration dependence, with increasing levels of amylin corresponding to increasing leakage of the membrane. When the negative head groups of the DOPS are replaced with DOPA (lacking a head group and holding a net negative charge) there is still a significant effect at even low concentrations.

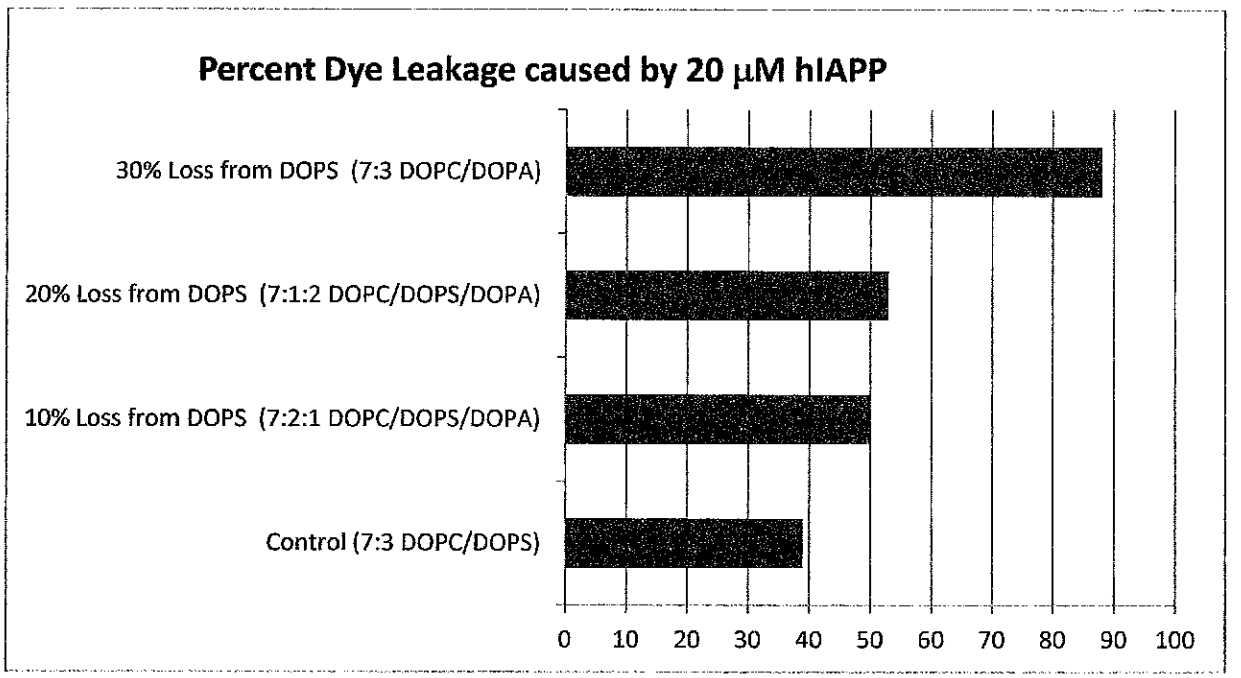
At the highest levels of alteration to the membrane (30% substitution with DOPA) in samples 3 and 6, there is heavy damage immediately to the membrane and complete destuction follows shortly afterwards with near 100% levels of dye leakage. It is important to note that in all three trials where the neutrally charged DOPC was replaced with DOPA there is nearly 100% leakage, further supporting not only the fact that head group loss is important, but that charge also has an impact on the toxic effects of amylin. Hopefully, future studies will hopefully be able to more accurately characterize the relationship between these two toxic factors.

Conclusion

Our hypothesis is clearly supported by the data. It is clear that the degree of amylin toxicity is significantly affected by the loss of head groups in the lipid membranes of pancreatic

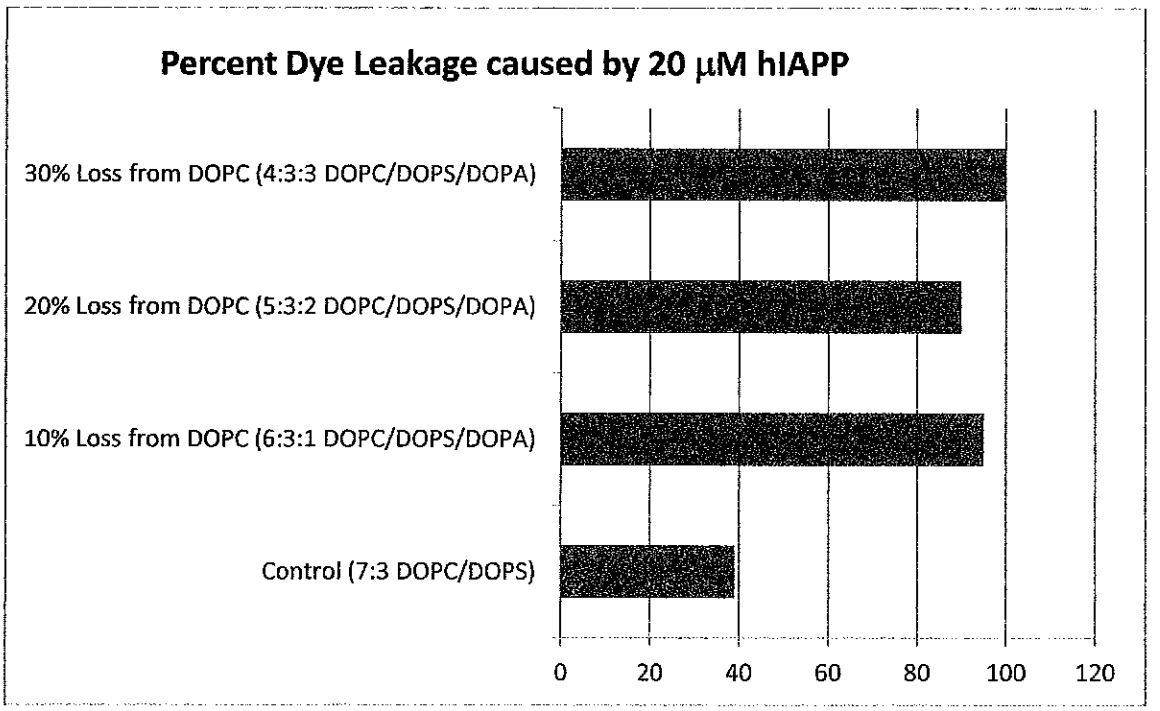
cells. In addition, the data show that the charge of the lipid bilayer also affects amylin toxicity. Depending on the head group of the lipids within the bilayer, the membrane can become more susceptible to damage. As shown below in graphs 8 and 9, as head groups are removed and the membrane becomes more negative, amylin has a deleterious effect on membrane stability.

Graph 8 clearly demonstrates that the percent of leakage increases from around 40% to roughly 50% with some head group loss from DOPS, and significantly increases to almost 90% with the loss of additional head groups. As the negatively charged lipid is replaced by a lipid with a larger negative charge, it is distinctly possible that not only the head group loss, but the increasingly negative charge of the lipid membrane has an effect on amylin toxicity.



Graph 8: 20μM Amylin percent leakage values as compared to control in samples 1-3

Graph 9 shows that as the neutral (and zwitterionic) DOPC loses its head group and becomes negatively charged, the lipid membrane immediately begins to catastrophically disintegrate. Even when only 10% of the membrane lipids lose head groups, the destruction of the membrane is near 100% when exposed to 20μM of amylin. This is significant not only for the loss of the head group, but in relation to the increasing negativity of the membrane. As the membrane becomes more negative, it is clear that amylin has in increasingly damaging effect on the membrane. The loss of head groups may be primarily responsible, but it would be impossible to attribute the increasing amount of membrane damage simply to a single element in the data. Perhaps with further and more rigorous testing the degree with which charge and head group loss affect membrane degradation can be both better characterized and understood.



Graph 9: 20μM Amylin percent leakage values as compared to control in samples 4-6

In our trials this was demonstrated through dye leakage, but in a normal human cell, any disruption of the membrane is likely to prove significant. The loss of head groups and replacement of a neutral charge with a net negative charge help to explain the increased amount of insulin resistance in Type II Diabetes patients as they age, as well as the decrease in overall insulin production, and is supported by previous studies of amylin. [11] As the β-cells produce insulin and amylin, they inadvertently produce the method of their destruction due to age-related changes in the very elements that are designed to protect the cell. While it cannot be said that amylin is wholly to blame for the increase in diabetic side effects with age, it certainly cannot be discounted. Further work must be done to characterize the exact behavior of amylin on living cells, as it is not likely that the effects shown in these trials will be exactly analogous in the human body. With more information and a better understanding of the complexities involved, some of the deleterious effects of amylin can be mitigated in Type II Diabetic patients of all ages.

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Supplemental Table 1: Raw fluorescence data used to calculate the membrane leakage fraction

[Table:TABLE M 485/528]								
Time	Control	100% Leakage	A3	A4	A5	A6	A7	A8
0:00:00	31	120	35	42	68	62	72	75
0:00:59	30	120	35	42	70	63	72	75
0:01:58	32	120	35	44	67	62	73	73
0:02:57	31	119	35	41	69	63	75	75
0:03:56	31	118	36	41	67	62	73	75
0:04:55	31	120	35	40	67	61	73	74
0:05:54	31	120	36	40	67	62	75	74
0:06:53	32	119	35	40	68	62	74	75
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0:08:51	31	120	35	41	69	61	75	75
0:09:50	32	117	36	41	68	61	75	74
0:10:49	32	118	35	40	67	62	74	75
0:11:48	32	117	35	41	69	62	75	74
0:12:47	31	118	35	41	67	61	75	74
0:13:46	31	117	35	41	67	63	76	75
0:14:45	32	118	36	41	69	62	76	75
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0:39:20	34	113	36	41	67	62	79	75
0:40:19	33	111	36	41	67	62	79	74

0:41:18	34	113	36	41	68	62	79	74
0:42:17	33	112	37	41	67	61	80	75
0:43:16	34	112	36	40	67	61	79	75
0:44:15	34	112	36	41	67	62	79	75
0:45:14	34	112	35	40	66	62	80	75
0:46:13	34	111	36	40	67	62	80	75
0:47:12	35	112	36	41	67	62	80	75
0:48:11	35	112	36	42	68	62	80	75
0:49:10	34	112	36	41	67	63	80	75
0:50:09	34	112	36	41	66	62	80	72
0:51:08	35	111	35	41	66	61	80	76
0:52:07	33	111	36	41	67	62	81	75
0:53:06	35	111	37	40	67	63	81	75
0:54:05	36	111	37	41	67	63	80	75
0:55:04	34	111	36	41	68	62	80	76
0:56:03	35	111	35	41	67	62	81	75
0:57:02	35	111	38	41	67	61	82	76
0:58:01	36	110	35	40	67	61	81	75
0:59:00	35	111	36	41	67	62	81	75
0:59:59	35	110	36	42	67	62	82	75
1:00:58	34	111	37	43	67	61	81	75
1:01:57	35	109	36	41	68	62	82	75
1:02:56	36	111	36	41	66	62	81	75
1:03:55	36	109	38	41	66	62	81	75
1:04:54	36	109	37	41	67	62	81	74
1:05:53	35	108	36	41	67	62	80	76
1:06:52	35	108	36	41	66	62	81	75
1:07:51	36	109	36	42	66	62	81	76
1:08:50	36	108	35	41	67	62	82	76
1:09:49	37	108	38	41	67	63	81	75
1:10:48	35	108	36	41	67	62	81	75
1:11:47	36	109	37	41	67	62	82	72
1:12:46	37	108	37	41	66	62	81	75
1:13:45	37	107	36	40	67	62	81	75
1:14:44	37	107	36	41	67	62	82	75
1:15:43	36	108	36	40	66	63	82	75
1:16:42	36	107	36	42	66	62	82	75
1:17:41	36	107	37	41	66	62	81	75
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1:19:39	38	107	37	41	67	62	80	75
1:20:38	37	107	37	41	65	64	81	75
1:21:37	37	107	37	40	65	62	83	75
1:22:36	37	107	36	42	66	61	81	76
1:23:35	37	107	37	42	66	62	82	76
1:24:34	36	106	36	41	67	63	82	75
1:25:33	37	105	37	41	69	63	83	75
1:26:32	38	106	36	41	67	62	81	76

1:27:31	37	105	35	41	67	63	82	75
1:28:30	38	106	37	40	66	63	83	76
1:29:29	36	105	36	41	66	62	82	74
1:30:28	39	105	36	42	67	61	83	75
1:31:27	37	105	37	41	66	62	82	75
1:32:26	39	106	37	41	67	62	83	75
1:33:25	39	106	37	41	67	63	82	75
1:34:24	38	106	36	40	67	63	82	75
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1:36:22	38	105	36	42	67	63	81	75
1:37:21	38	105	36	41	65	62	83	75
1:38:20	38	105	37	41	65	61	82	75
1:39:19	37	104	37	40	66	61	82	74
1:40:18	37	104	37	42	67	61	83	75
1:41:17	40	105	37	41	66	63	83	75
1:42:16	39	105	38	42	66	62	82	75
1:43:15	38	104	36	41	67	60	83	76
1:44:14	39	104	37	40	65	63	83	75
1:45:13	38	104	37	40	65	62	82	75
1:46:12	39	103	35	42	68	62	82	75
1:47:11	39	103	37	40	66	61	83	76
1:48:10	40	102	36	42	67	62	83	76
1:49:09	39	103	37	40	65	63	83	77
1:50:08	38	103	38	41	66	62	83	74
1:51:07	39	103	36	41	66	62	83	75
1:52:06	38	104	37	41	67	62	84	75
1:53:05	40	102	37	42	64	63	82	76
1:54:04	40	103	36	41	67	62	83	75
1:55:03	40	103	38	41	67	62	84	75
1:56:02	39	102	38	42	66	62	83	75
1:57:01	40	103	37	40	67	62	84	75
1:58:00	39	102	38	41	65	62	83	75
1:58:59	39	103	36	41	66	62	83	75
1:59:58	41	103	37	42	66	62	83	77
2:00:57	41	103	38	41	67	63	84	75
2:01:56	39	102	38	39	66	61	83	76
2:02:55	40	103	37	41	66	62	83	74
2:03:54	39	102	37	42	65	63	84	76
2:04:53	40	102	37	42	67	62	83	75
2:05:52	40	102	38	42	67	63	83	75
2:06:51	40	101	37	41	66	61	83	76
2:07:50	39	102	37	41	66	61	82	74
2:08:49	40	102	37	41	65	62	84	75
2:09:48	40	102	37	40	66	63	83	75
2:10:47	40	102	38	41	66	63	82	75
2:11:46	40	101	39	42	67	63	84	76
2:12:45	40	102	37	41	67	63	83	75

2:13:44	41	105	37	41	67	63	83	75
2:14:43	40	102	38	41	66	63	82	76
2:15:42	39	101	37	41	66	63	83	75
2:16:41	41	101	37	42	67	62	82	75
2:17:40	41	101	37	41	65	61	82	74
2:18:39	40	100	37	41	66	62	84	76
2:19:38	41	101	38	44	66	62	84	76
2:20:37	41	102	38	42	66	62	83	75
2:21:36	41	101	37	42	66	62	83	75
2:22:35	39	101	37	40	66	62	84	74
2:23:34	42	101	37	41	67	62	83	75
2:24:33	41	100	38	41	66	62	83	77
2:25:32	41	101	36	42	67	63	83	75
2:26:31	41	100	37	42	67	62	82	75
2:27:30	40	101	38	41	66	63	84	74
2:28:29	42	101	37	41	66	62	83	75
2:29:28	41	100	37	42	66	62	83	74
2:30:27	42	100	38	41	65	63	83	75
2:31:26	41	99	37	42	66	62	83	76
2:32:25	41	100	37	44	65	63	82	75
2:33:24	41	100	37	42	66	62	84	75
2:34:23	42	100	37	42	65	63	83	74
2:35:22	41	101	39	41	65	62	83	75
2:36:21	42	101	38	42	66	63	83	76
2:37:20	42	99	39	42	66	62	83	74
2:38:19	42	100	37	41	66	62	83	75
2:39:18	41	99	38	39	67	61	83	76
2:40:17	41	98	39	42	66	62	83	75
2:41:16	42	101	36	42	66	64	82	74
2:42:15	43	100	41	41	66	62	83	76
2:43:14	42	100	38	41	65	61	82	75
2:44:13	41	99	38	42	67	63	83	76
2:45:12	42	100	37	42	66	61	84	78
2:46:11	41	99	38	42	66	62	84	74
2:47:10	42	100	38	41	67	62	83	76
2:48:09	43	103	39	41	65	63	83	74
2:49:08	42	99	38	41	66	62	83	75
2:50:07	42	99	37	41	65	63	83	76
2:51:06	42	97	37	41	66	62	82	75
2:52:05	43	101	38	41	66	63	84	75
2:53:04	42	99	37	42	66	62	83	75
2:54:03	42	100	38	41	66	61	82	75
2:55:02	43	99	38	41	66	63	83	76
2:56:01	43	99	38	41	65	62	81	75
2:57:00	42	99	38	41	66	62	83	74
2:57:59	43	99	39	41	66	63	84	75
2:58:58	42	102	38	40	66	63	83	74

2:59:57	42	100	38	41	66	64	83	75
Average	37.1902	107.0326	36.6467	41.1033	66.5870	62.1141	80.8641	75.0109
Amylin Concentration (μM)	0	0	0.5	1	2.5	5	10	20
Fraction Leaked	0	1	-0.0078	0.056	0.4209	0.3569	0.6253	0.5415
Percent Leaked	0.00	100.00	-0.78	5.60	42.09	35.69	62.53	54.15
[Table:TABLE M 485/528]								
Time	Control	100% leakage	B3	B4	B5	B6	B7	B8
0:00:00	28	108	35	39	64	60	67	65
0:00:59	29	108	35	39	65	56	67	66
0:01:58	29	108	35	39	64	59	67	67
0:02:57	29	107	36	39	65	59	68	66
0:03:56	28	108	36	39	64	60	67	67
0:04:55	28	108	35	39	63	60	68	67
0:05:54	30	108	35	39	64	59	68	65
0:06:53	27	109	35	38	64	59	69	65
0:07:52	29	106	35	38	64	59	67	66
0:08:51	29	108	34	39	63	60	69	66
0:09:50	29	107	35	39	64	60	68	66
0:10:49	28	107	35	38	63	59	68	66
0:11:48	28	108	34	38	64	60	68	66
0:12:47	28	108	35	38	64	59	69	67
0:13:46	29	107	36	38	64	61	69	66
0:14:45	28	107	35	38	64	59	68	67
0:15:44	29	108	34	38	63	59	69	66
0:16:43	29	107	34	38	63	59	71	66
0:17:42	29	106	35	37	63	59	70	67
0:18:41	29	107	35	39	64	59	70	66
0:19:40	29	106	35	38	63	58	69	66
0:20:39	29	107	34	39	63	60	70	66
0:21:38	28	107	34	38	64	60	70	67
0:22:37	27	107	34	37	63	60	70	66
0:23:36	28	106	34	38	64	60	71	66
0:24:35	29	106	34	37	63	59	71	67
0:25:34	29	106	33	38	63	60	71	67
0:26:33	29	107	35	38	61	60	71	68
0:27:32	29	105	35	38	64	60	71	66
0:28:31	29	106	35	38	63	59	72	67
0:29:30	30	105	35	37	62	59	72	67
0:30:29	29	106	38	37	64	60	71	67
0:31:28	29	105	34	38	62	58	71	67
0:32:27	29	105	34	39	63	59	71	66
0:33:26	29	106	35	38	62	59	72	67

0:34:25	30	105	35	38	62	60	72	67
0:35:24	29	105	33	38	64	59	71	67
0:36:23	29	106	34	38	62	57	73	67
0:37:22	29	105	36	39	62	59	71	66
0:38:21	29	106	34	39	63	60	71	67
0:39:20	30	105	33	37	62	60	72	66
0:40:19	28	105	34	39	63	59	73	69
0:41:18	30	105	35	37	63	58	72	67
0:42:17	29	105	35	37	62	59	73	67
0:43:16	29	105	35	37	63	59	72	67
0:44:15	30	104	35	37	63	60	73	67
0:45:14	29	106	35	38	63	59	73	67
0:46:13	29	103	35	38	62	59	73	67
0:47:12	30	103	36	38	62	60	72	66
0:48:11	31	104	34	38	63	59	73	68
0:49:10	29	104	34	37	63	59	73	67
0:50:09	29	105	35	37	62	59	73	67
0:51:08	29	104	34	39	62	59	73	67
0:52:07	30	105	33	38	63	59	73	67
0:53:06	29	105	34	37	63	59	74	66
0:54:05	29	104	34	38	63	60	73	69
0:55:04	30	104	34	37	62	59	73	66
0:56:03	29	103	33	37	63	59	74	67
0:57:02	29	104	33	37	63	58	73	67
0:58:01	31	103	35	39	62	58	75	67
0:59:00	29	105	35	38	62	59	74	67
0:59:59	30	104	34	37	63	59	73	67
1:00:58	30	104	34	37	62	59	74	66
1:01:57	29	103	34	38	62	59	75	66
1:02:56	29	103	34	38	62	59	76	67
1:03:55	29	104	35	37	63	59	74	67
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1:05:53	30	104	36	37	62	60	73	67
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1:07:51	30	103	35	38	62	59	75	67
1:08:50	30	103	34	37	62	60	75	67
1:09:49	30	103	34	36	62	59	74	67
1:10:48	31	103	35	37	63	59	74	67
1:11:47	29	103	35	37	61	58	74	67
1:12:46	30	103	35	38	61	59	75	67
1:13:45	30	103	34	36	61	59	74	66
1:14:44	30	103	36	38	61	59	75	67
1:15:43	32	102	33	38	61	59	75	67
1:16:42	31	102	36	38	62	59	76	67
1:17:41	31	102	34	38	62	59	75	67
1:18:40	31	102	34	38	62	59	74	66
1:19:39	30	103	34	37	61	59	74	67

1:20:38	30	103	35	38	61	60	75	67
1:21:37	30	102	33	37	62	61	75	66
1:22:36	31	101	34	37	62	60	76	66
1:23:35	30	101	35	37	61	59	75	67
1:24:34	30	102	35	37	62	58	75	67
1:25:33	31	104	34	39	62	58	75	67
1:26:32	32	102	35	37	61	59	74	66
1:27:31	32	103	35	37	60	58	76	67
1:28:30	30	102	34	39	61	59	75	68
1:29:29	31	104	35	38	61	58	76	67
1:30:28	30	101	34	36	61	58	76	68
1:31:27	31	101	36	37	62	58	75	66
1:32:26	31	102	34	37	62	59	75	67
1:33:25	31	101	34	37	61	59	74	66
1:34:24	31	104	35	37	61	59	77	67
1:35:23	30	101	33	39	61	60	75	68
1:36:22	31	101	35	37	60	59	74	67
1:37:21	31	102	35	36	61	59	75	67
1:38:20	31	102	35	37	62	59	76	67
1:39:19	31	103	35	36	61	59	75	66
1:40:18	31	101	34	37	61	59	76	68
1:41:17	31	102	35	38	63	59	75	67
1:42:16	31	101	34	36	61	58	75	67
1:43:15	31	102	35	37	61	59	75	67
1:44:14	31	101	35	37	61	59	74	66
1:45:13	30	101	34	37	62	59	74	66
1:46:12	31	100	34	37	58	59	75	67
1:47:11	31	101	35	36	61	59	76	67
1:48:10	31	102	34	38	62	58	75	66
1:49:09	31	102	35	38	62	60	73	67
1:50:08	30	100	35	36	60	58	75	68
1:51:07	31	101	35	38	63	59	75	67
1:52:06	32	101	34	36	61	59	75	67
1:53:05	31	102	34	36	61	59	75	67
1:54:04	31	101	35	38	62	59	75	66
1:55:03	32	100	34	37	61	59	74	66
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1:57:01	31	101	35	39	60	59	76	67
1:58:00	31	100	35	38	62	59	76	67
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1:59:58	32	101	34	37	61	59	75	67
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2:03:54	31	101	35	37	61	57	76	66
2:04:53	32	100	34	38	62	60	76	66
2:05:52	32	101	35	39	63	58	76	67

2:06:51	31	100	34	38	61	58	76	67
2:07:50	31	99	34	38	61	58	75	67
2:08:49	31	101	36	39	61	58	76	67
2:09:48	31	100	35	37	61	59	76	67
2:10:47	31	101	35	37	61	58	76	66
2:11:46	32	100	33	37	62	59	75	66
2:12:45	32	99	34	38	61	58	75	67
2:13:44	32	101	34	38	61	58	76	69
2:14:43	31	99	35	37	62	58	76	67
2:15:42	32	99	33	38	60	59	76	66
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2:26:31	31	99	34	37	60	58	76	68
2:27:30	32	100	36	38	60	58	76	67
2:28:29	31	100	35	37	60	58	75	66
2:29:28	33	100	31	38	59	58	75	67
2:30:27	32	99	33	37	61	58	76	67
2:31:26	32	100	34	37	61	58	75	69
2:32:25	32	98	34	36	60	58	77	67
2:33:24	32	99	34	37	60	58	75	67
2:34:23	32	98	35	37	60	59	76	66
2:35:22	31	98	34	37	61	58	76	65
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2:38:19	32	99	35	38	59	58	75	65
2:39:18	32	98	34	38	60	58	76	68
2:40:17	33	99	34	38	60	58	76	66
2:41:16	32	99	34	37	60	58	75	66
2:42:15	31	98	34	37	60	58	76	67
2:43:14	33	98	35	37	60	59	76	67
2:44:13	32	99	34	38	60	59	76	67
2:45:12	32	99	34	37	60	57	75	67
2:46:11	33	99	34	37	60	58	76	67
2:47:10	32	100	34	37	59	58	76	67
2:48:09	33	100	34	37	60	58	77	67
2:49:08	33	99	35	38	60	58	75	67
2:50:07	33	96	35	38	60	58	76	67
2:51:06	32	98	35	38	59	58	76	66
2:52:05	32	97	35	38	59	58	76	67

2:53:04	33	98	33	38	59	58	75	66
2:54:03	33	100	35	38	60	58	75	68
2:55:02	33	97	34	37	60	57	76	67
2:56:01	33	98	35	37	59	57	76	66
2:57:00	33	98	35	37	60	58	76	67
2:57:59	32	99	35	37	58	59	76	66
2:58:58	33	98	35	38	59	58	76	66
2:59:57	33	98	34	37	60	59	76	67
Average	30.5380	102.3859	34.4946	37.5978	61.6250	58.8043	73.8370	66.7446
Amylin Concentration (μM)	0	0	0.5	1	2.5	5	10	20
Fraction Leaked	0	1	0.0551	0.0983	0.4327	0.3934	0.6026	0.5039
Percent Leaked	0.00	100.00	5.51	9.83	43.27	39.34	60.26	50.39
[Table:TABLE M 485/528]								
Time	Control	100% leakage	C3	C4	C5	C6	C7	C8
0:00:00	27	115	33	36	59	58	67	70
0:00:59	28	114	34	35	59	56	69	70
0:01:58	28	115	32	36	60	55	68	70
0:02:57	29	114	32	35	60	55	69	69
0:03:56	28	114	32	35	59	55	68	70
0:04:55	29	114	32	35	58	56	69	70
0:05:54	29	115	32	36	58	55	68	69
0:06:53	28	114	32	35	59	55	69	71
0:07:52	29	113	32	36	59	54	69	69
0:08:51	29	112	32	36	58	55	69	70
0:09:50	30	113	32	36	59	55	70	69
0:10:49	29	113	32	37	59	55	71	68
0:11:48	29	113	32	35	59	56	71	69
0:12:47	31	113	33	34	59	55	71	67
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0:14:45	30	113	32	35	59	55	70	70
0:15:44	30	113	32	35	58	55	71	69
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2:58:58	38	103	35	37	58	55	73	67
2:59:57	38	103	34	34	57	55	73	68
Average	34.1793	107.6413	32.4348	35.5054	58.3696	55.2011	73.7935	68.6413
Amylin Concentration (μM)	0	0	0.5	1	2.5	5	10	20
Fraction Leaked	0	1	-0.0237	0.0181	0.3293	0.2862	0.5392	0.4691
Percent Leaked	0.00	100.00	-2.37	1.81	32.93	28.62	53.92	46.91
Sample 1	37.19	107.03	36.65	41.10	66.59	62.11	80.86	75.01
Sample 2	30.54	102.39	34.49	37.60	61.63	58.80	73.84	66.74
Sample 3	34.18	107.64	32.43	35.51	58.37	55.20	73.79	68.64
Average from all runs	33.97	105.69	34.53	38.07	62.19	58.71	76.16	70.13
Amylin Concentration (μM)			0.50	1.00	2.50	5.00	10.00	20.00
Fraction Leakage			0.01	0.06	0.39	0.34	0.59	0.50
Standard Deviation	3.33	2.87	2.11	2.83	4.14	3.46	4.07	4.33
Standard Deviation as a % of avg.	0.10	0.03	0.06	0.07	0.07	0.06	0.05	0.06