Poly-ion Layers in Layer by Layer Coating of Bacteria: Growth and Zeta Potential

by
Emma N Latta

A THESIS

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degree of

Honors Baccalaureate of Science in BioHealth Sciences
(Honors Associate)

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Abstract approved:_____

Linda Blythe

To determine the best poly-ion layers to be used in encapsulation of pyrrolizidine alkaloid degrading anaerobic microbes, a study was carried out to test three layers on coating ability and ability of the bacteria to grow after being coated. Two positive layers, poly(allylamine hydrochloride) (PAH) and poly-L-lysine, and one negative layer, humic acid (HA), were selected for testing. PAH and HA were hypothesized to be the best pairing of positive and negative layers due to their common use in thin layer films. Coating ability was measured using zeta potential, and ability to grow after being coated was measured by spectrometry. PAH and HA coated cells showed a charge change between layers for the first four layers and then stopped changing. This indicates they stopped coating the cells after four layers. Cells coated with Poly-L-lysine and HA showed charge change for all six tested layers, indicating they coated the cells for all six layers. PAH-coated cells didn't show growth over a 48 hour period. Poly-L-lysine and HA coated bacteria showed growth after 48 hours. These results prove the hypothesis null and indicate that poly-L-lyisne and HA are the best layers for use in encapsulating pyrrolizidine alkaloid degrading anaerobic microbes.

Key Words: poly-ion, poly(allylamine hydrochloride), poly-L-lysine, humic acid, pyrrolizidine alkaloids, layer by layer

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Emma N Latta, Author

Introduction

Pyrrolizidine alkaloids (PAs) are found in about 3% of the world's flowering plants (Culvenor 1980). PA-containing plants are part of the Boraginaceae, Compositae, and Leguminosae plant families (Cheeke 1998). In the northwest United States, tansy ragwort (*Senecio jacobaea*, part of the Compositae family) is the main source of PA poisoning in livestock. The main PAs in tansy ragwort are macrocyclic compounds such as senecionine. There are multiple paths of PA metabolism in the liver, but the most prominent is dehydrogenation to pyrroles (Mattocks 1986). Pyrroles are the main cause of tansy ragwort's hepatoxic effects. Due to their high reactivity and bioactivation in the liver, pyrroles can form covalent bonds with DNA, causing liver diseases such as swelling of hepatocytes, centrilobular necrosis, and eventually loss of liver function (Cheeke 1998). On the United States west coast, biological control of tansy ragwort involves the cinnabar moth (*Tyria jacobaeae*), the ragwort flea beetle (*Longitarsus jacobaeae*), and the ragwort seed fly (*Botanophila seneciella*). This method of control has reduced losses for the cattle industry by 75% since 1970 (Coombs et al 1996). Even with a large decrease of tansy ragwort infestation due to biological control methods, PA poisoning still costs Oregon agriculture about $20 million annually (Craig 1991). Cows and horses who consume about 5-10% of their body weight in tansy ragwort plant material develop terminal liver disease (Craig 1979). Sheep have been shown to be significantly more resistant, and are able to eat 200% to 3001% of their body weight in tansy ragwort before experiencing any severe effects of PA poisoning (Craig 1986, Hooper 1978).

Originally, the source of the different species susceptibility in tolerance was believed to be in the way sheep and cattle livers process PAs (Mattocks 1986). In 1986 however, Craig et al postulated that the difference was due to microbial populations in the rumen, and not differing liver metabolism. To separate PA metabolism in the liver from the rumen, PA extract from tansy ragwort was injected into the liver of sheep via the ruminal vein, while others received an oral dose. Sheep injected with the PA extract showed toxic changes to their histopathologic profile while those taking the PA extract orally did not (Craig et al 1986). The results of the study indicated that the rumen played a role in the sheep's resistance to PA toxicosis. Further research was able to isolate a consortium of bacteria able to degrade PAs (Lodge-Ivey 2005). Using molecular cloning techniques and denaturing gradient gel electrophoresis, researchers determined the consortium to contain no more than five bacterial species associated to the *Anaerovibrio*, *Desulfovibrio*, *Megasphaera*, *Prevotella*, and *Synergistes* generas (Rattray 2006, Lodge-Ivey 2005). The consortium was named L4M2 and has been used in subsequent studies on PA degradation by ruminal microbes.

The research presented in this paper furthers the effort to create a probiotic to protect cattle from the harmful effects of tansy ragwort using L4M2. This encapsulation method could also be used to transfer hexahydro-1,3,5,-trinitro-1,3,5-triazine (RDX)-degrading microbes between different ruminants. RDX is a commonly used explosive which can contaminate drinking water

underneath soil beds due to improper disposal or production (U.S. Environmental Protection Agency 2017). Cool-season grasses can take up explosives from the soil allowing an animal with RDX degrading microbes in its rumen to degrade the explosives (Duringer et al 2010). As all of the bacteria in L4M2 and RDX degrading consortia are anaerobic, they need protection from oxygen while being transferred. Currently, the goal is to use poly-ion layers to accomplish oxygen protection. Three layers commonly used to make layer-by-layer films were selected to be tested for their layering ability, as measured by zeta potential; and ability for bacteria to grow after being coated, as measured by optical density. These layers were two positive poly-ions, poly(allylamine hydrochloride) (PAH) and poly-L-lysine, and one negative poly-ion, humic acid (HA). PAH is one of the most studied poly-ions in layer-by layer (LBL) films, while poly-L-lysine has been successfully used in biological applications such as encapsulation of pancreatic islets for transportation (Shao et al 2010, Mendelsohn et al 2003, Schneider et al 2001). HA is used in the creation of thin film sensors (Yuan et al 2013, Crespilho et al 2005). All three layers were tested for their performance using zeta potential testing, and for growth after the coating treatment.

Hypothesis

HA as the negative layer and PAH as the positive layer are hypothesized to be able to adequately coat the bacteria without causing adverse effects.

Methods

Selecting and Maintaining Model Microbes

Anaerovibrio liptolyticus was selected as a representative microbe for L4M2 for growth tests, as it is known to be in the L4M2 consortium and able to degrade RDX (Eaton et al 2013, Lodge-Ivy 2005). *A. liptolyticus* is strictly anaerobic and grows on complex media[a]. Media was dispensed into balch-type tubes in an anaerobic glove bag[b]. Culture was transferred at a ratio of 1 mL culture to 9 mL reduced complex media.

Lysis can occur when *A. lioptolyticus* is exposed to oxygen which would affect zeta potential results. So, instead *E. coli* was selected as a representative microbe for zeta potential. *E. coli* is a facultative anaerobe allowing LBL to be performed on the bench top instead of in the anaerobic hood, and provides a more accurate zeta potential reading. *E. coli* was grown at ambient conditions on LB media[c] and transferred using aseptic technique.

All cultures were incubated at 37˚C with gentle agitation. Maintenance cultures were incubated for 24 hours and then transferred.

General LBL Methodology

One and a half mL of culture was placed into each microcentrifuge tube. Microcentrifuge tubes were centrifuged for 5 minutes at 8,000*g* then supernatant fluid was removed and discarded. One mL sterile 0.85% saline was added to all tubes and vortexed. Tubes were then centrifuged for 5 minutes at 8,000*g*, then supernatant fluid was removed and discarded. This saline wash process was repeated for a second time, and one tube was set to the side as the zero layer sample. One mL positive layer was added to test tubes and 1 mL sterile saline was added to control tubes. All tubes were vortexed, left to sit for 15 minutes, and then centrifuged for 5 minutes at 8,000*g*. Supernatant fluid was removed and discarded. One mL of sterile saline was added to all tubes and vortexed. Tubes were centrifuged for 5 minutes at 8,000*g* and supernatant fluid was removed and discarded. This process of adding a layer and then washing the cells was repeated until the desired number of layers was achieved. The first layer was always negative, then layers alternated between positive and negative layers as layers were added. The last layer added is the outermost layer and should match the charge of the cell. Layers were made at a ratio of 2 mg poly-ion to 1 mL ultra-pure water. Poly-ion layers were gassed with CO_2[d] and autoclaved[e] at 121˚C for 20 minutes.

Bacterial Growth After Coating with Poly-ions

The ability of bacteria to grow after being coated with the positive layer was tested by putting one positive layer on the sample using the general LBL methodology described above. The ability of bacteria to grow after being coated with the negative layer was tested by putting one positive and one negative layer on the sample. The negative layer sample had the negative layer as its outermost layer but also had one positive layer because LBL must always start with a positive layer. Therefore if the positive layer used is shown to hinder bacterial growth, the negative layer cannot be assessed on its ability to allow the bacteria to grow because lack of growth could be due to the positive layer and not the negative layer. Two samples for each test were made (two PAH, two PAH/HA, two poly-L-lysine, and two poly-L-lysine/HA). The positive control was transferred to a fresh tube of complex media and the negative control consisted of autoclaved *A. liptolytica* culture. Both the positive and negative controls were washed as in the beginning of the LBL process. All samples were then transferred into separate tubes of complex media. Growth was measured in optical density at 600 nm on a spectrometer[f] at 0, 24, and 48 hours. The spectrometer's zero measure was set using complex media.

Zeta Potential

The electrical potential difference existing between the solid particles and bulk of the conducting liquid is defined as the zeta potential. This measurement was used to determine if the LBL was effectively coating the bacterial cells. A good positive coating would be represented by a high zeta potential compared to the control and a good negative coating would be represented by a low zeta potential.

Samples were prepared using the general LBL method, with one sample being put to the side after the supernatant was removed from every wash step (second wash step for Layer 0). Control samples used 0.85% sterile saline solution instead of poly-ion layers with one sample taken off as described above. Three samples for each layer were taken as described above for the two different poly-cations. Samples were then stored at 4 ˚C until use. Zeta potential was measured using a zetasizer[g]. Samples were suspended in 1.5 mL of 0.85% sterile saline before being put into a folded capillary cell. The folded capillary cells were washed with ultra-pure water twice between samples. Three measures were taken for each sample.

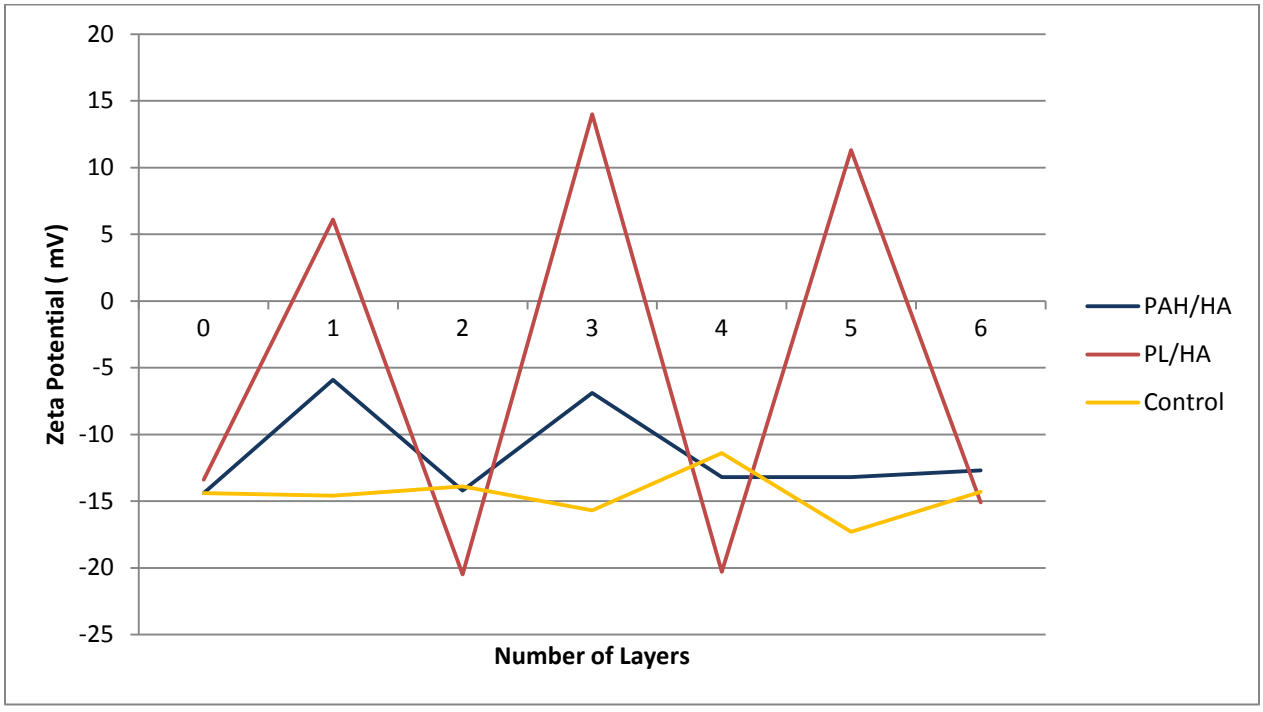
Statistical Analysis

Statistical analysis of the data was done using a paired *t*-test using Excel[h].

Results

Figure 1

Zeta potential of poly(allylamine hydrochloride) (PAH) and poly-L-Lysine (PL) with humic acid (HA) on *E. coli* as compared to control



A difference in charge was seen for the first four layers of the PAH/HA layer pairing (Figure 1, blue line) as compared to the control (Figure 1, yellow line). Layers 4 and 5 showed no difference in charge (Figure 1, blue line). At no point did we see the PAH layer create a positive charge on the cell, indicating that although there appears to be some coating, it is not strong.

There is a negative charge on the cells when HA is used, indicating some layering of *E. coli*. There was a drastic difference in charge between all layers of the poly-L-lysine/HA pairing (Figure 1, red line). A positive charge was observed during the poly-L-lysine layer indicating an effective coat was created. The HA layers have a negative charge, indicating a layer of HA was present. Precise numerical values for zeta potential can be found in Appendix 1.

The positive controls showed growth after 24 hours and the negative controls showed no growth over the 48 hour period. This confirms that the bacteria were growing normally without the coating, and that there was no contamination during the coating process. *A. lyptolyticus* coated with PAH had no measurable growth after 48 hours, but in fact showed a significant decrease in optical density (Table 1, $P<0.01$). This decrease was due to oxidative additions in the media which changed the color of the media over time. *A. liptolyticus* coated with poly-L-lysine showed no growth after 24 hours but showed growth after 48 hours (Table 2, $P=0.02$).

Table 1

Optical density measurements for PAH and HA on *A. liptolyticus*

Time (hr)	PAH Layer A	PAH Layer B	HA Layer A	HA Layer B	Positive Control	Negative Control
0	.156	.124	.155	.116	.129	.109
24	.091	.074	.088	.054	.362	.069
48	.086	.050	.080	.049	.319	.058

Table 2

Optical density measurements for PL and HA on *A. liptolyticus*

Time (hr)	Poly-L-Lysine Layer A	Poly-L-Lysine Layer B	HA Layer A	HA Layer B	Positive Control	Negative Control
0	.266	.212	.287	.248	.191	.143
24	.255	.184	.237	.202	.534	.088
48	.636	.616	.668	.660	.514	.082

Discussion

The main purpose of this study was to determine the best positive and negative poly-ion layers for coating bacteria using a layer-by-layer method. Coated anaerobic bacteria from a sheep's rumen could then be transferred through the environment to other species' gastrointestinal systems. PAH and HA were hypothesized to be the best positive and negative layers, respectively, for coating bacteria due to their frequent use in creating thin layer films (Yuan 2013, Shao. 2010, Crespilho 2005, Mendelsohn. 2003). Poly-L-lysine was also tested due to its use as a thin film in biological applications (Schneider 2001). Layers were tested based on their ability to: 1) coat the bacteria; and 2) ability of the bacteria to grow after being coated. Coating ability was measured using zeta potential, while bacterial growth was measured using optical density. Our hypothesis was found to be null. PAH was not the best positive layer for coating bacteria when measured using zeta potential but poly-L-lysine was. On average, a charge

difference of 7.5 mV was seen between the first four layers before a leveling out was seen in the PAH/HA samples (Figure 1, Appendix 1). This is not the most optimal charge difference, as compared to the average charge difference between all layers for poly-L-lysine/HA samples which was 29 mV. This clearly indicates poly-L-lysine and HA were better at coating the bacteria than PAH and HA. PAH was also not the best at allowing bacterial growth after coating. Bacteria did not grow over a 48 hour time frame after being coated with PAH, while the positive control showed growth after 24 hours. Once again, poly-L-lysine was found to be a better material for the positive layer, as growth was seen after a 48 hour time period. While this demonstrates a longer lag period than the positive control which showed growth after 24 hours, this is still a positive result as the cells were able to grow to the same level as the control. This difference in lag time could be due to the time it takes for the bacteria to free themselves from the layers coating them. In theory, this could cause them to be unable to grow with too many layers, as they would no longer be able to free themselves. Further testing in this area could determine the optimal number of layers which can be added before the cells are unable to grow out of them.

The overarching purpose of this study was to further research into the creation of a probiotic for cattle which will protect against PA poisoning. PA poisoning has a significant impact on the livestock industry despite the use of biological control agents such as the cinnabar moth (Craig 1991). Previous research found that sheep have a significantly higher tolerance to PAs than cattle and horses due to differing microbial communities in their rumen (Craig et al 1986). This lead to the idea of transferring the microbes involved in PA tolerance from sheep to cattle in the form of a probiotic. The first step in the development of the probiotic was to determine the microbes involved in PA tolerance and characterize them. A consortium able to degrade PAs was isolated and determined to contain no more than five bacterial species associated to the *Anaerovibrio*, *Desulfovibrio*, *Megasphaera*, *Prevotella*, and *Synergistes* generas by use of molecular cloning techniques and denaturing gradient gel electrophoresis (Rattray 2006, Lodge-Ivey 2005). The research described here leads into the next step of continuing this work, namely developing a technique for protecting the anaerobic consortium from oxygen so that it can be easily moved in the environment. It has been found that one should be able to protect the bacteria from oxygen using the poly-ion layers tested here. In this study, the best pairing of positive and negative layers was determined to be poly-L-lysine and HA. Future research into the creation of a probiotic for cattle and horses will test if these layers can protect the bacteria from oxygen through in vivo studies.

Concurrent research in the lab has been looking into other detoxifying abilities of sheep ruminal microbes and, notably, their ability to degrade explosives such as RDX. RDX is a toxin to a wide range of plants and mammals and is classified as a group C possible human carcinogen (U.S. Environmental Protection Agency 2017). Improper disposal and production of RDX materials as well as contamination due to testing threatens drinking water supplies under contaminated soil beds (U.S. Environmental Protection Agency 2017). Researchers found that

cool-season grasses were able to take up explosives from the soil and store them; and that sheep ruminal bacteria were able to degrade RDX in about 4 days (Eaton et al 2011, Duringer et al 2010). This lead to formation of the idea of Phyto-Ruminal-Bioremediation: a technology for the cleanup of toxic compounds by first pulling them up into plants and then degrading them by ruminants and their microbes that eat the plants. Phyto-Ruminal-Bioremediation is more cost effective than other methods of munitions clean up, making it a feasible option particularly for developing countries. Of the microbes found to degrade RDX, *A. liptolyticus* was identified as one of the better bacterial degraders (Eaton et al 2013). Thus, combining the munitions research with the concurrent research for creation of a probiotic to transfer ruminal microbes is possible. This would allow for use of other, possibly native, ruminants in the cleanup of contaminated areas, other than just sheep. Bacteria capable of degrading explosives could be transferred to ruminants more common to other areas of the globe such as goats and camels using the probiotic. This would allow Phyto-Ruminal-Bioremediation to be more accessible to places outside of the United States of America which have been contaminated by explosives.

This paper presents research findings that could be used in multiple applications and as a basis for further research. First and foremost, research into the ability of poly-ion layers to protect anaerobic bacteria from oxygen should be performed. Once the method for creating the probiotic has been completed, it can be used for more than just protection from PA poisoning. It can be used to remove human toxins such as explosives from contaminated areas by Phyto-Ruminal-Bioremediation, using animal species native to the area being decontaminated. As we learn more about the power of ruminal microbes, more applications for this research will reveal themselves.

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Appendix 1

Zeta potential measurements for *E. coli* cells coated with poly(allylamine hydrochloride) and humic acid as well as *E. coli* cells coated with poly-L-lysine (PL) and humic acid. The first layer is a poly-cation, the second layer is a poly-anion, with layers continuing to alternate between poly-cation and poly anion.

Layer #	PAH 1 (mV)	PAH 2 (mV)	PAH 3 (mV)	PL 1 (mV)	PL 2 (mV)	PL 3 (mV)	Control (mV)
0	-12.6 -14.4 -15.3 **Average** **-14.1**	-12.7 -13.8 -14.2 **Average** **-13.6**	-15.6 -14.5 -15.9 **Average** **-15.3**	-14.4 -15.5 -16.5 **Average** **-15.5**	-10.2 -12.5 -13.1 **Average** **-11.9**	-11.6 -12.8 -12.5 **Average** **-12.8**	-13.8 -14.2 -15.3 **Average** **-14.4**
1	-7.05 -8.55 -8.41 **Average** **-8.00**	-6.99 -8.55 -11.4 **Average** **-8.98**	-0.645 -0.426 -1.25 **Average** **-0.774**	6.27 5.94 6.40 **Average** **6.20**	5.65 4.92 6.54 **Average** **5.70**	5.83 6.61 6.58 **Average** **6.34**	-13.4 -14.9 -15.6 **Average** **-14.6**
2	-10.4 -12.3 -11.7 **Average** **-11.5**	-16.0 -18.1 -15.1 **Average** **-16.4**	-15.0 -13.9 -15.4 **Average** **-14.8**	-18.9 -19.7 -19.5 **Average** **-19.4**	-22.2 -23.3 -24.1 **Average** **-23.2**	-18.4 -18.7 -19.2 **Average** **-18.8**	-12.9 -14.1 -14.7 **Average** **-13.9**
3	-3.65 -8.06 -8.41 **Average** **-6.86**	-4.57 -5.03 -6.21 **Average** **-5.27**	-8.30 -8.31 -9.03 **Average** **-8.55**	11.6 12.3 15.5 **Average** **13.1**	13.0 13.3 13.7 **Average** **13.3**	15.6 14.4 16.5 **Average** **15.5**	-13.5 -15.5 -18.2 **Average** **-15.7**
4	-10.7 -11.5 -12.6 **Average** **-11.6**	-16.2 -18.2 -19.4 **Average** **-17.9**	-9.28 -10.3 -10.7 **Average** **-10.1**	-16.9 -17.4 -19.4 **Average** **-17.9**	-22.1 -24.8 -26.0 **Average** **-24.3**	-17.6 -18.1 -20.2 **Average** **-18.6**	-10.9 -11.7 -11.5 **Average** **-11.4**
5	-11.7 -12.0 -12.3 **Average** **-12.0**	-9.65 -14.9 -15.4 **Average** **-13.3**	-14.3 -15.0 -13.7 **Average** **-14.3**	13.4 14.3 13.8 **Average** **13.8**	12.1 11.7 14.3 **Average** **12.7**	8.26 8.63 4.87 **Average** **7.25**	-14.6 -18.4 -18.9 **Average** **-17.3**
6	-11.2 -9.77 -11.4 **Average** **-10.8**	-12.2 -12.5 -14.5 **Average** **-13.1**	-15.3 -17.7 -18.5 **Average** **-14.3**	-10.4 -16.4 -21.9 **Average** **-16.2**	-13.5 -16.3 -16.3 **Average** **-15.4**	-13.2 -12.4 -15.5 **Average** **-13.7**	-13.6 -14.5 -14.9 **Average** **-14.3**

Each sample was measured three times and then the average was taken.

Footnotes

a. c. 580 mL water, 400 mL clarified ovine rumen fluid, 1.0 mL resazurin, 4.0 g sodium carbonate, 4.0 g cellobiose, 2.0 g trypticase, 1.1 g sodium citrate dihydrate, 1.0 g yeast extract, 0.6 g sodium chloride, 0.6 g ammonium sulfate, 0.6 g potassium phosphate monobasic, 0.2 g sodium acetate, 0.12 g magnesium sulfate heptahydrate, 0.079 calcium chloride dehydrate, 0.048 sodium propionate, and 0.027 g sodium butyrate

b. Coy Laboratory Products Inc Grass Lake, MI USA

c. LB media was made using Difco LB broth, Miller (Luria-Bertani) mix from Becton, Dickinson and Company. Sparks, MD USA

d. Industrial Welding Corvallis, OR USA

e. Consolidated Boston, MA USA

f. Spectronic 20D. Milton Roy Company. Rochester, NY USA

g. Malvern Panalytical, Malvern UK.

h. Microsoft Redmond, WA USA