Comparison of novel silver plated magnetic particles versus commercially available magnetic particles by chemiluminescent assay.

Zhelev P[1], He J[2], Allard M[1], Thorogood S[2], Carville D1, Gawad Y[1].

INTRODUCTION

Paramagnetic particles (MPs) are the most widely used solid support in automated clinical lab immunoanalyzers(1), taking advantage of their large surface area and high level of automation(2). In the quest for improved immunoassay sensitivity and system performance, most developers employ light as the signaling mechanism in the form of chemiluminescence(1)(3)(4).

Current trends in optimizing immunoassay tests on automated platforms employ various optimization methods for improved light output by employing antibodies with improved characteristics, improved light emitting material, assay conditions and conjugation procedures tailored to the paramagnetic particles. Further, the use of polymer-coated paramagnetic particles of ideal size with a high amount of surface functional groups/smaller parking area have been demonstrated to improve the test performance.

This approach worked very well with all the enzyme assays generating color-based results, since these are the first applications requiring latex or paramagnetic particles as a solid surface in heterogeneous immunoassays. However, currently used paramagnetic particles were not historically optimized for light detection, being the largest source of light signal loss in binding assays that employ them.

Higher light output from the assays allow faster and more sensitive detection of very limited amounts of the analytes. However, commercial sources of MPs employ black/brown magnetic pigment which usually adsorb a large portion of the generated light. A new generation of light emitting detection systems require a novel approach, not only with the instrumentation design and test parameters, but also with the solid support for the binding assays. Newly-developed silver-coated paramagnetic particles are addressing the current problems by increasing the efficiency of light harvesting caused by light scattering effect from MPs, therefore, minimizing the signal loss and improving the detection limits by reflecting the light. In this study, we compared the performance of the newly developed silver-coated paramagnetic beads to commercial MPs.

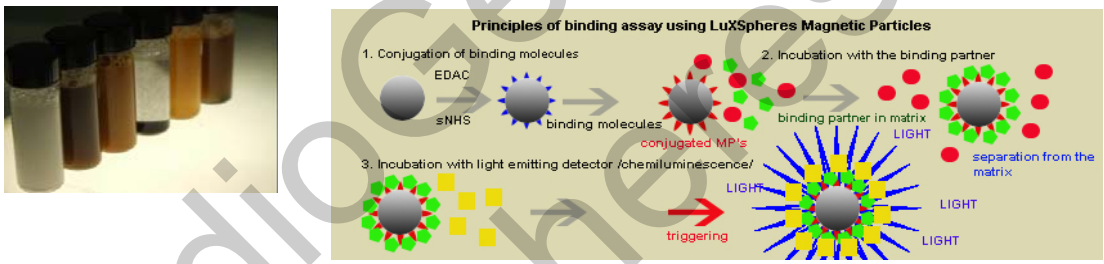

Fig 1. Luxspheres silver plated magnetic particles (MPs) (first) visual comparison with standard MPs used as a solid surface in binding assays today.

MATERIALS AND METHODS

Materials

All the paramagnetic particles were received as free samples from the manufacturers in order to participate in the comparison trial involving chemiluminescence and bioluminescence. All the paramagnetic particles were presenting carboxyl functionalities for further conjugation of proteins. The size of the paramagnetic particles varies between the manufacturers:

Manufacturer name	Size	Cat Number
Spherotech	3.0-3.9 µm	CMS-30-10
Seradyn	0.886 µm	45152105050250
Seradyn	1.371 µm	65152105050250
Magsphere	17 µm	MAG1476
Magsphere	1.8 µm	MCU608015
Polymerlabs	2.7 µm	LodeStar 2.7 Carboxyl
Estapor	2.3 µm	M1 200/20
Estapor	1.4 µm	M1 070/60
Ademtech	0.292 µm	0213
Ademtech	0.213 µm	0212
Ademtech	0.503 µm	0215
Luxspheres	8 um	GF232-LX

Table 1. MPs used in the comparison

N-Cyclohexyl-*N'*-(β-[*N*-methylmorpholino]ethyl)carbodiimide *p*-toluenesulfonate salt (CMC), *N*-Hydroxysulfosuccinimide sodium salt (sulfo NHS), Rabbit Anti-Goat Horseradish Peroxidase (HRP) conjugate (H and L specific), Casein (vitamin free), 3,3′,5,5′-Tetramethylbenzidine (TMB) Liquid Substrate System for ELISA were all purchased from Sigma Aldrich Canada (Oakville, ON).
Sulfo-SMCC Sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane-1-carboxylate (Sulfo SMCC), 2-Iminothiolane•HCl (Traut's Reagent), Streptavidin Horseradish Peroxidase (HRP) conjugate , BCA Protein Assay Kit were purchased from Pierce (Rockford, IL).
Sephacryl S200 was purchased from Amersham Biosciences.
Lumigen PS Atto (reduced intensity) was purchased from Lumigen Inc.(Southfield, MI).
Goat IgG and Donkey Anti-Goat (affinity purified) were purchased from Lampire Biologicals (Pipersville, PA).
Other reagents were of analytical reagent or laboratory grade.
Deionized, distilled water form Millipore system Direct-Q3 was used in all procedures.

Methods

Conjugation of goat IgG to paramagnetic particles

- A standard two-step conjugation procedure of the antibody to the carboxyl group on the paramagnetic particles employing carbodiimide/NHS chemistry was employed. MPs were prepared with a first activation step followed by a conjugation step as follows:
- MPs from all suppliers were adjusted to 20 mg/ml (except Ademtech MPs 15mg/ml) in volume of 1 ml.
- All samples were washed consequently with 0.01 M NaOH once, H_2O twice and 0.05 M KH_2PO_4twice.
- MPs were separated from the washing solution by magnet.
- Washing was followed by the activation step.
- MPs were activated by 0.0276 M sulfo-NHS and 0.0236 M CMC in 0.05 M KH_2PO_4 pH 6.0. for 1 hour incubation at room temperature (RT) with constant rotation.
- MPs were washed three times with 0.05 M KH_2PO_4 pH 6.0.
- 0.2 mg Goat IgG in 1 ml of 0.0375 M Borate, 0.05 M NaCl pH 8.5 was immediately introduced.
- After 2 hour incubation at RT, MPs were washed with three volumes of 0.01 M PBS
- MPs were quenched for 30 min RT incubation with 0.01 M ethanolamine (0.01 M PBS, pH 7.4) with constant rotation.
- Blocking of the beads was completed by 1% Casein in 0.01 M PBS pH 7.4 for an hour RT rotation.

Preparation of Donkey Anti-Goat conjugates

The bioluminescent protein Obelin was employed for light measurements. Conjugation procedure of IgG and obelin was previously described (5). Briefly, SMCC-activated Donkey Anti-Goat IgG was incubated with previously thiolated Obelin by Traut's reagent (SH-obelin) at a molar ratio of 1:10 for overnight at 4 C. The obtained conjugate was purified by gel filtration on Sephacryl S200 column (Amersham Biosciences) equilibrated with 0.2M NaCl, 0.005M EDTA, and 0.05 M MOPS, pH 7.1. Protein concentration was determined by BCA Protein Assay Kit (Pierce).

Beads Testing

Evaluation of MPs by an ELISA employed colorimetric testing with TMB and light emitting tests that employ either chemiluminescent material Lumigen PS Atto (reduced intensity substrate) or bioluminescence as follows:

A. Evaluation by ELISA employing Colorimetric testing with TMB

- A 0.25 % MPs already conjugated with Goat IgG MPs were prepared in 0.2 ml of 0.05M Tris, 0.14M NaCl, 0.125 % Tween 20, 0.005 % Pluronic F68, pH 7.8 (Washing buffer).
- Washing buffer was removed by magnet.
- 0.2 ml in duplicates from serial dilutions 1:40k, 1:80k and 1:160K of Rabbit Anti-Goat HRP conjugate in 0.05M Tris, 0.14M NaCl, 0.125 % Tween 20, 0.005 % Pluronic F68, 0.5 % Casein (Reaction buffer) were applied.
- Incubation for 30 min at RT with shaking (maximum speed 10 on Lab Line instruments titter plate shaker).
- MPs were washed two times with washing buffer and transferred to white strips (Greiner Maximum Binding, flat bottom White*).
- Two more washing steps were applied in the wells before applying the substrate.
- To the each well, 0.150 ml of diluted substrate 1:2 TMB in 0.05 M Phosphate-Citrate buffer pH 5.0 was applied.
- Development time was 5 min and 0.1 ml of already developed substrate without magnetic particles was transferred to transparent plate (BD Falcon).
- The reaction was stopped with 0.1 ml 0.5M H_2SO_4.
- The measurement was done with a plate reader at 450 nm (ThermoMax, Molecular Devices).
- Background of the MPs was evaluated by 1:40K Streptavidin-HRP (since the testing procedure is half-sandwich type).

B. Evaluation of MPs by Chemiluminescent testing

Procedure is the same as for the colorimetric testing except:

- The Rabbit Anti-Goat HRP conjugate dilutions are from 1:160K, 1:320k and 1:640k and used substrate is Lumigen PS Atto (reduced intensity).
- The measurement took place with the particles on board in the white strips.
- 0.05 ml of mix from Lumigen PS Atto (A) and (B) (mix 1:1) were applied to each well.
- The peak signal was collected for 1 sec by Luminoscan Ascent Thermoelectron.
- Background of the MPs was evaluated by 1:320K Streptavidin-HRP (since the testing procedure is half-sandwich type).

C. Evaluation of the amounts of MPs on harvested light by Chemiluminescent testing

Procedure is the same as in B above except:

- The Rabbit Anti-Goat HRP conjugate dilution is constant 1:160 k.
- Concentration of MPs is 0.125% , 0.0625%, 0.0313% and 0.0156%.

D. Evaluation of MPs by Bioluminescent light measurements

MPs conjugated to Goat IgG were evaluated by an ELISA using Donkey Anti-Goat-Obelin as follows**:**

- 0.25 % of Goat IgG conjugated MPs were prepared in 0.2 ml of 0.0074M Na_2HPO_4, 0.0025M KH_2PO_4, 0.25M NaCl, 0.005 M EDTA, 0.005 M Mercaptoethanol, 0.25 % Tween 20, 0.005 % Pluronic F68, 0.02 M N-Acetyl Cystein, pH 7.4(Washing buffer).
- Washing buffer was removed by magnet and two hundred 0.2 ml in duplicates from serial dilutions 8, 4 and 2 µg/ml of Donkey Anti-Goat-Obelin conjugate in 0.0074M Na_2HPO_4 , 0.0025M KH_2PO_4 , 0.25M NaCl, 0.050 M EDTA, 0.005 M Mercaptoethanol, 0.25 % Tween 20, 0.005 % Pluronic F68, 0.02 M N-Acetyl Cysteine, 0.5 % Casein (Reaction buffer) were applied.
- Incubation for 30 min at RT with constant shaking (maximum speed 10 on Lab Line instruments titter plate shaker).
- MPs were washed two times with washing buffer and transferred to white strips (Greiner Maximum Binding, flat bottom, White*).
- Two more washing steps were applied in the wells before measuring the signal, 0.05 ml of washing buffer was added to each well after final washing step.
- Light emission was triggered by injecting 0.05 ml of 0.1 M $CaCl_2$, 0.05 M MES; pH6 in Luminoscan Ascent Thermoelectron.
- Signal was collected for 30 sec and integration was applied (area under the curve).
- Since the procedure for testing is half sandwich type, background of the MPs was evaluated by 4 µg/ml Streptavidin-Obelin.

RESULTS

- All the measurements were performed in duplicate and the average value was assigned for every point.
- Although the paramagnetic beads from various suppliers were of different sizes, no adjustment for surface area was used.
- Colorimetric ELISA was used as a reference method since no impact of MP color is expected.
- Light measurements were evaluated by 2 different methods, chemiluminescence and bioluminescence.
- All the results from the ELISA by both color and light measurements are presented in the tables/ figures 2, 3, 4 and 5.

Dilution RAG-HRP	Magnetic particles			
	GF232 LX	Spherotech	Polymer Labs /LODE Star	Seradyn 651
	OD mean	OD mean	OD mean	OD mean
1 : 40 k	2.15	3.81	2.55	3.39
1 : 80 k	1.45	3.46	2.03	2.85
1 : 160 k	1.00	3.04	1.31	1.78
0	0.04	0.04	0.04	0.15

Dilution RAG-HRP	Magnetic particles			
	Estapor M1 200/20	Estapor M1 070/60	Ademtech 0212	Ademtech 0213
	OD mean	OD mean	OD mean	OD mean
1 : 40 k	3.17	2.86	3.09	2.92
1 : 80 k	2.98	2.25	2.66	2.31
1 : 160 k	2.25	1.67	1.86	1.58
0	0.06	0.05	0.12	0.15

Dilution RAG-HRP	Magnetic particles			
	Magsphere MAG1476	Seradyn 451	Ademtech 0215	Magsphere MCU608015
	OD mean	OD mean	OD mean	OD mean
1 : 40 k	1.92	3.15	2.90	3.06
1 : 80 k	0.98	2.71	2.68	1.96
1 : 160 k	0.62	2.01	1.64	1.17
0	0.04	0.05	0.10	0.26

Table 2. Comparative Enzyme Linked Immunosorbent Assay employing TMB

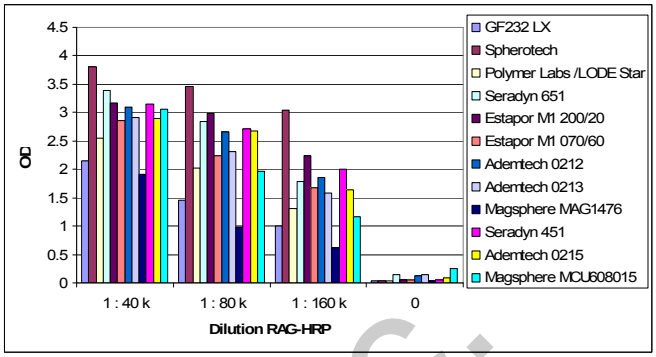

Fig 2. Colorimetric signal from MPs generated by Enzyme Linked Immunosorbent Assay employing TMB

Dilution RAG-HRP	Magnetic particles			
	GF232 LX	Spherotech	Polymer Labs /LODE Star	Seradyn 651
	RLU mean	RLU mean	RLU mean	RLU mean
1 : 160 k	2.15E+07	7.13E+06	3.28E+06	2.79E+06
1 : 320 k	1.48E+07	3.38E+06	2.79E+06	1.97E+06
1 : 640 k	1.03E+07	1.79E+06	1.74E+06	1.07E+06
0	3.43E+04	5.21E+03	3.80E+03	1.61E+03

Dilution RAG-HRP	Magnetic particles			
	Estapor M1 200/20	Estapor M1 070/60	Ademtech 0212	Ademtech 0213
	RLU mean	RLU mean	RLU mean	RLU mean
1 : 160 k	4.09E+06	7.12E+06	3.09E+06	1.36E+06
1 : 320 k	3.64E+06	5.76E+06	1.62E+06	1.25E+06
1 : 640 k	2.45E+06	3.86E+06	8.97E+05	6.88E+05
0	1.20E+04	3.33E+04	5.07E+03	6.47E+03

Dilution RAG-HRP	Magnetic particles			
	Magsphere MAG1476	Seradyn 451	Ademtech 0215	Magsphere MCU608015
	RLU mean	RLU mean	RLU mean	RLU mean
1 : 160 k	5.19E+06	9.58E+05	2.24E+06	1.31E+06
1 : 320 k	2.90E+06	5.95E+05	1.40E+06	5.26E+05
1 : 640 k	1.67E+06	3.35E+05	7.50E+05	2.49E+05
0	3.14E+03	6.69E+02	1.96E+03	3.79E+03

Table 3. Comparative Enzyme Linked Immunosorbent Assay employing Lumigen PS Atto

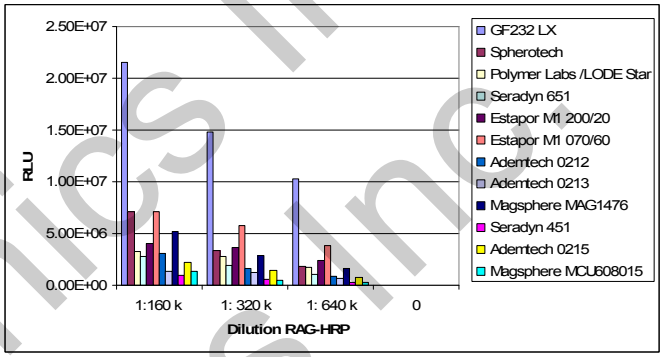

Fig 3. Light signal ("glow" type) from MPs generated by Enzyme Linked Immunosorbent Assay employing Lumigen PS Atto

DAG-Obelin (µg/ml)	Magnetic particles			
	GF232 LX	Spherotech	Polymer Labs /LODE Star	Seradyn 651
	RLU mean	RLU mean	RLU mean	RLU mean
8	8.02E+07	3.18E+07	1.85E+07	1.53E+07
4	5.12E+07	1.62E+07	8.41E+06	7.72E+06
2	2.71E+07	7.80E+06	4.00E+06	3.50E+06
0	5.20E+04	2.90E+04	1.64E+03	9.81E+02

DAG-Obelin (µg/ml)	Magnetic particles			
	Estapor M1 200/20	Estapor M1 070/60	Ademtech 0212	Ademtech 0213
	RLU mean	RLU mean	RLU mean	RLU mean
8	1.49E+07	1.37E+07	1.03E+07	7.86E+06
4	8.07E+06	6.76E+06	4.82E+06	2.64E+06
2	4.07E+06	3.08E+06	2.51E+06	1.37E+06
0	5.43E+04	6.53E+04	7.92E+03	4.81E+03

DAG-Obelin (µg/ml)	Magnetic particles			
	Magsphere MAG1476	Seradyn 451	Ademtech 0215	Magsphere MCU608015
	RLU mean	RLU mean	RLU mean	RLU mean
8	6.60E+06	6.02E+06	3.55E+06	2.60E+06
4	3.59E+06	3.02E+06	2.23E+06	1.51E+06
2	1.67E+06	1.54E+06	1.18E+06	7.59E+05
0	1.86E+03	3.31E+02	8.02E+03	1.48E+04

Table 4. Comparative Bioluminescent Linked Immunosorbent Assay

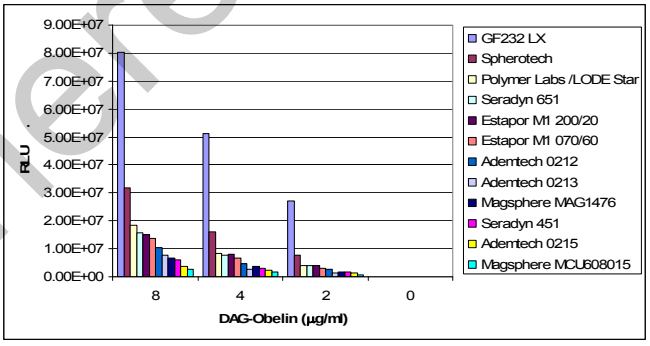

Fig 4. Light signal ("flash" type) from MPs generated by Bioluminescent Linked Immunosorbent Assay

Dilution of MPs RAG-HRP 1: 160k	Magnetic particles			
	GF232 LX	Spherotech	Polymer Labs /LODE Star	Seradyn 651
	RLU mean	RLU mean	RLU mean	RLU mean
0.1250 %	2.25E+07	1.01E+07	4.76E+06	6.42E+06
0.0625 %	1.39E+07	1.54E+07	7.82E+06	1.27E+07
0.0313 %	8.23E+06	2.06E+07	1.16E+07	2.01E+07
0.0156 %	4.61E+06	2.06E+07	1.16E+07	1.45E+07

Dilution of MPs RAG-HRP 1: 160k	Magnetic particles			
	Estapor M1 200/20	Estapor M1 070/60	Ademtech 0212	Ademtech 0213
	RLU mean	RLU mean	RLU mean	RLU mean
0.1250 %	9.00E+06	1.27E+07	3.08E+06	1.65E+06
0.0625 %	1.34E+07	1.52E+07	6.51E+06	4.24E+06
0.0313 %	1.45E+07	7.88E+06	1.03E+07	7.97E+06
0.0156 %	1.09E+07	4.45E+06	9.08E+06	8.55E+06

Dilution of MPs RAG-HRP 1: 160k	Magnetic particles			
	Magsphere MAG1476	Seradyn 451	Ademtech 0215	Magsphere MCU608015
	RLU mean	RLU mean	RLU mean	RLU mean
0.1250 %	5.31E+06	2.91E+06	1.22E+06	1.18E+06
0.0625 %	4.51E+06	6.28E+06	2.84E+06	1.21E+06
0.0313 %	2.89E+06	1.05E+07	6.83E+06	1.07E+06
0.0156 %	2.32E+06	1.30E+07	8.64E+06	5.15E+05

Table 5. Effect of the MPs amount on Enzyme Linked Immunosorbent Assay employing Lumigen PS Atto

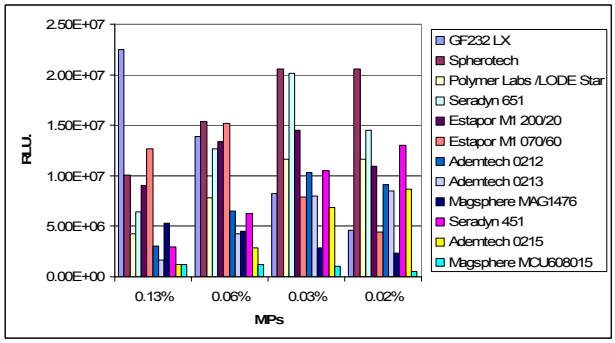

Fig 5. Effect of the MPs amount on light signal from Enzyme Linked Immunosorbent Assay employing Lumigen PS Atto

CONCLUSION

Our data demonstrate the enhanced light harvesting effect of the silver-coating of paramagnetic particles in comparison with the darker color magnetic pigment. Although commercial particles of various sizes and density of carboxyl groups were tested, the silver-coated paramagnetic particles show a 3-7 fold improvement in the collected light signal over the dark-colored magnetic pigment embedded in paramagnetic particles from various commercial sources by both chemiluminescence ("glow" type) and the referent bioluminescence ("flash" type) (Fig. 3, Fig 4.). The improved light harvesting effect of silver coating was demonstrated also when various amounts of MPs were employed. Even though the amount of conjugated Goat IgG to the Silver-plated MPs is considerably lower in comparison with commercial counterparts, based on colorimetric measurements (Fig. 2), the improvement in the measured light signal was evident, regardless of the amount of generated light. A clear correlation exists between the amount of paramagnetic material used and the collected light signal indicating that the major effect is due to the light harvesting effect rather than antibody density. This will facilitate enhancing the sensitivity of tests that employ them. Since the light loss due to paramagnetic particles is the major factor contributing to assay sensitivity, it seems that employing these newly-developed particles would be a good strategy for improving assay sensitivity. Although it is evident that the developed silver coated MPs will improve the collected light signal by the nature of their color, improving the density of their surface functional groups will further improve their light harvesting effect.

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[1]CardioGenics Inc., Mississauga, ON, and
[2]LuXSpheres Inc., Mississauga, ON