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13. ABSTRACT (Maximum 200 words)

Our program has focused on the use of dendritic polymers as potential enhancers of biodetection or biosensor schemes for the detection of analytes such as biological warfare agents. Dendritic polymers exhibit a new macromolecular architecture, and novel properties as well, when compared to conventional polymers such as linear polymers. The aim was to see if we could utilize these new polymers for improved design of biosensors. To this end we investigated the utility of attaching antibodies to a solid phase (such as a QCM device) via polyamidoamine (PAMAM) dendrimers in an effort to improve parameters such as sensitivity in immunoassays. Clinical diagnostic assays in the commercial sector have successfully incoroporated these polymers for improved performance, and we chose to see if similar advantages could be translated to alternative transducers such as a QCM device. In addition, preliminary investigations were initiated in which PAMAM dendrimers are used as chemical amplifiers in biosensors in which detection is done colorimetrically via the naked eye. We also began to investigate immunoreactions and dendrimer-enhanced immunoprecipitations in capillaries as an alternate platform for a biodetection device.

14. SUBJECT TERMS
dendritic polymers, dendrimers, biological warfare agents, chemical warfare agents

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DENDRITIC POLYMERS: A NEW CLASS OF POLYMERS FOR DECONTAMINATION AND DETECTION OF CHEMICAL AND BIOLOGICAL WARFARE AGENTS

FINAL PROGRESS REPORT

Herbert M. Brothers II and Donald A. Tomalia

January 15, 1999

U.S. ARMY RESEARCH OFFICE

DAAH04-95-1-0652

MICHIGAN MOLECULAR INSTITUTE

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Michigan Molecular Institute
Grant Number: DAAH04-95-1-0652

H.M. Brothers II and D.A. Tomalia

15 January 1999

2. Statement of Research Problem and Relevance to the Army

Research for the improvement and/or development of biodetection devices remains highly relevant to the Army as rogue nations and terrorist groups continue to design and deploy strategic biological weapons. The U.S. armed forces and citizenry both at home and abroad remain threatened by the deployment and use of such weapons of mass destruction. Development of methods for rapid and reliable detection of chemical and biological agents is paramount for proper actions to be taken in the operational theater or for proper emergency responses to be initiated for public safety. Although there are numerous sensor technologies and prototypes, biosensors (and chemical sensors) in general continue to have several shortcomings such as low sensitivity, poor selectivity, incapability of real-time continuous monitoring, and poor reliability. Technology improvement in any of these areas brings a practical and field usable biosensor closer to reality.

Our research program is investigating the utility of dendritic polymers in the design of novel sensor systems and in the improvement of existing systems. Dendritic polymers are the fourth major class of macromolecular architecture and exhibit novel properties when compared to their classical counterparts such as linear, branched, and cross-linked polymers. Since the discovery of dendritic polymers in the late 1970's, over two thousand papers have been published on the design of new dendritic materials, analysis of dendritic structures, and more recently, development of applications. The majority of these papers have been published within the last five years. The interest in dendritic polymers by the scientific community remains high, and applications utilizing these materials are only beginning to become apparent.

It is our aim to improve the efficacy of sensors based on immunoreactions (and potentially other specific ligand-receptor interactions) by incorporating dendritic polymers (1,2) into biosensor device construction. Dendritic polymers have found recent success in the commercial sector of immunodiagnostics. It is our intent to transfer the scientific principles that have improved commercial immunodiagnostic instrumentation to devices which may be useful for improved detection of biological warfare agents. In the past year, we have continued to focus on the development and improvement of processes for the synthesis of dendritic polymer-antibody constructs for potential use in

biodetection and biosensor devices. Earlier in the research program, we developed methods for the synthesis, purification and characterization of dendrimer-antibody conjugates. We then took these conjugates and tested them in a prototype continuous flow immunosensor in which signal transduction was through a quartz crystal microbalance (QCM). We also compared the performance of the dendrimer-antibody conjugates to the performance of antibodies that were immobilized to the QCM surface with polyethleneimine or protein A. More recently, our conjugation technology has been transferred to scientific personnel at ERDEC as part of an effort to test these conjugates in other biodetection schemes. This technology transfer has resulted in an increased effort to refine the process technology associated with the production, purification and characterization of dendrimer-antibody conjugates, and to this end, we have dedicated the majority of our most recent efforts in this direction.

3. Scientific Progress and Accomplishments (Summary of Results)

(1) *Dendrimer-antibody conjugate process and analytical research.*

We continue to work with Dade-Behring (formerly Dade International which was formerly Baxter Diagnostics) in the development of polyamidoamine dendrimer (PAMAM) – antibody constructs for use in the commercial sector of clinical diagnostics.(3-5) Successful commercialization of the Dade-Behring instrument which incorporates these conjugates was successfully launched into the marketplace in 1998. Dade Behring's instrument is the Stratus® CS and is shown in Figure 1. The role of dendrimer-antibody conjugates in this instrumentation is schematically depicted in Figure 2. The Stratus CS instrument is designed to be placed at point of care locations (such as emergency rooms) to rapidly diagnose patients for myocardial infarction. The instrument is capable of rapidly analyzing and quantifying levels of creatinine kinase muscle bone (CKMB), troponin I, and myoglobin proteins in the blood serum of suspected heart attack patients. In the past year, we have largely **focused on improved synthesis and analysis of dendrimer-antibody constructs** for potential use in other, yet similar, applications such as biodetection devices and sensors.(6,7) To this end, we have found the following:

- Dendrimer-antibody conjugates can now be made in relatively high yield (e.g., relative conjugate yields can approach 90%) with absolute conjugate yields (i.e., conjugate recovered / total starting material of protein) approaching 80%. These yields are obtained when synthesizing conjugates on the ca. 1 mg scale. Better absolute yields should be obtainable when scaling up such reactions. A schematic showing one type of conjugation chemistry is shown in Figure 3. We have achieved good yields with other conjugation chemistries as well.

- In conjunction with synthesis improvements, we have worked on methods for better purification of dendrimer-antibody constructs. We typically have used ion-exchange chromatography on carboxymethyl cellulose to remove unconjugated dendrimer and unconjugated antibody (if either is present in an immunoassay system, data interpretation may be complicated by potential interferences such as nonspecific binding or reduced

sensitivity). In place of ion-exchange chromatography on carboxymethyl cellulose, we have tried sulfoxyethyl cellulose, protein A/G affinity resin and ultrafiltration through 100k MWCO Centricon devices to remove unconjugated dendrimer. The ion exchange resin methods seem to remain the most efficient way of simultaneously removing unconjugated antibody and unconjugated dendrimer. Some conjugate products have shown contamination with dendrimer when using ion-exchange chromatography, but this problem appears to be alleviated when reducing the dendrimer excess in conjugation reaction mixtures. In addition, column dimensions may play an important role in the purity of conjugates obtained when chromatographed on carboxymethyl cellulose resin.

- In an effort to better characterize dendrimer-antibody constructs, we have tried analyzing such constructs using MALDI-TOF MS (matrix assisted laser desorption ionization – time of flight – mass spectroscopy). Preliminary data indicates that the conjugates are heterogeneous and are not easily analyzed by this technique. Spectra obtained from the starting antibody exhibit interpretable data, however.

- We have improved our analysis of dendrimer-antibody constructs by utilizing SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis) procedures in addition to analysis by native PAGE. Analysis of dendrimer-antibody conjugates can more easily be done on SDS-PAGE gels owing to the higher availability of commercial gels which exhibit suitable data. Native gels which are suitable for analysis of dendrimer-antibody constructs are no longer easily obtainable. Figure 4 shows such a gel and the differences in migration behavior of the dendrimer-antibody conjugates and unconjugated dendrimer and unconjugated antibody.

(2) *Detection of immunoreactions utilizing capillary electrophoretic instrumentation.*

Another field we started to investigate is that of using capillary electrophoretic instrumentation in the detection of environmental antigens. This is a particularly exciting area of investigation, since the technique of detecting immunoreactions in capillaries should be able to performed quickly (nearly real time results), can be done with very little reagent consumption (nearly a "reagentless" detector) and can be done repeatedly (nearly capable of continuous monitoring). In addition, capillary electrophoretic instrumentation is portable and can be placed in discrete locations (e.g, ships, subway stations, base camps, vehicles, etc.). The general concept of one form of capillary immunodetection is shown in Figure 5. In summary, the concept is to fill a capillary with buffer solution which has been saturated with air from the environment (e.g., a bubbler). Such filling operations are routine and automated in commercially available instruments. The volume of buffer required to fill a capillary is in the microliter range. Then, a plug of an antibody (nanoliters) is injected into one end of the capillary. The antibody plug is pumped through the capillary via electroosmotic flow. If the antibody encounters antigens (previously pumped in from the environment) that it is specific for, then an immunocomplex is formed which will have a different electrophoretic mobility than the antibody. This difference in electrophoretic mobility between the antibody and the antibody-antigen immunocomplex results in a distorted peak as the antibody passes the detector. The extent of peak distortion depends upon the concentration of antigen in the capillary tube.

We performed some preliminary experiments using BSA (bovine serum albumin) as a model soluble antigen and anti-BSA antibody as the detection reagent. Figure 6 shows capillary electropherograms from measurement of 10 μg / mL, 1 μg / mL, and 100 ng / mL concentrations of BSA within the capillary tube. The capillary was filled with buffer containing BSA as the analyte. An anti-BSA plug was injected, and its peak recorded as it passed a UV detector at 214 nm. The dotted lines in the figure are traces resultant from injecting an anti-BSA plug into the capillary without any BSA present. From this technique, we could clearly detect BSA down to a concentration of 1 μg / mL. Peak shape algorithms could be used to quantify the concentration of the antigen when it is unknown (such as in a sample from the environment). Attempts at detecting the BSA at 100 ng / mL have so far been unsuccessful. No dendritic polymers have been incorporated into this research yet, but they have the potential to act as signal amplifiers (i.e., increased effective molar absorptivity of the detection antibody) for increased sensitivity.

(3) *Use of dendrimers as signal amplifiers in tab immunoassays.*

We have done a bit more work on a project initiated last year which utilizes PAMAM dendrimers conjugated to a detection antibody rather than a capture antibody.(7) The general concept is shown in Figure 7. Here, we hoped to use the multiplicity of surface groups on dendrimers to act as an amplifier for a chromophore or other signal generator in lieu of an enzyme or other commonly used detection moiety typically found in immunoassay schemes. Previously, we tried using the dendrimer to noncovalently pick up bromphenol blue dye such that a positive immunoreaction could visually be detected by the naked eye (e.g., a blue spot would indicate a positive result). Although the general concept seemed to work, it was difficult to avoid nonspecific interactions where the amino-surfaced dendrimer nonspecifically interacted with the solid phase and/or the capture antibody. If nonspecific interactions are present, false positives may be obtained.

In an effort to remedy the problem we prepared a detection antibody conjugate in which 75% of the surface groups on the dendrimer were hydroxy groups and 25% were amino groups. The hope was that the neutrally charged hydroxy groups would not participate or contribute toward nonspecific binding. These dendrimers (with 75% hydroxy surface groups) do not pick up bromphenol blue as efficiently as the dendrimers with 100% amino surface groups, so we decided to try staining the dendrimer with gold after formation of the immunocomplex on a nitrocellulose tab. The dendrimer with 75% hydroxy surface groups stains well with gold, but in the tab immunoassay test, nonspecific binding was still observed with this system.

An alternative to having the dendrimer noncovalently pick up bromphenol blue or staining the final immunocomplex with gold, is to covalently attach a dye to the dendrimer which is attached to the detection antibody (i.e., formation of a ternary conjugate). We have synthesized such a conjugate by attaching a caproic acid derivative of fluorescein to a mixed surface dendrimer (75% OH surface groups) and attaching the dye-labelled dendrimer to the detection antibody. The concept is identical with that shown in Figure 7, but now the dye is covalently attached to the surface rather than

noncovalently attached. We have only tested the feasibility of synthesizing this type of conjugate and to date have not tried any immunoreactions with these conjugates.

(4) *Investigation of dendrimers as enzyme stabilizers: potential use for stabilization of enzymes used for decontamination.*

In addition, we spent some time this past year investigating the possibility of stabilizing enzymes with dendritic polymers. Funding for these studies was through a local company at no cost to the grant. We studied interactions of PAMAM dendrimers with alpha-chymotrypsin and horseradish peroxidase as model enzymes. In the presence of dendrimers, both enzymes did retain catalytic activity, even when covalently coupled to dendrimers, but our studies exhibited no strong evidence of stabilization of these particular enzymes when mixed or conjugated to PAMAM dendrimers. There was some evidence that the horseradish peroxidase was more thermally stable than the unconjugated enzyme when it was conjugated to a PAMAM dendrimer. Further studies should be done with enzymes that are relative to the interests of the Army since different enzymes will probably behave differently owing to differences in the tertiary structures and surface properties of the proteins.

(5) Significant results from prior research years.

In addition to what is stated above, the following accomplishments have been achieved over the grant period.

Results below are relevant to detection schemes. The goal was to incorporate dendritic polymers into biodetection schemes either as (1) an anchoring moiety for a bioreceptor or (2) as an amplifier for attachment of multiple signal generators.

- Successfully prepared covalent complexes between dendrimers and antibodies.

- Developed procedures to characterize and purify dendrimer-antibody conjugates.

- PAMAM dendrimers with primary amino surface groups form a rugged attachment to a noble metal surface without the need to form an initial self assembled monolayer.

- Demonstrated that a dendrimer-antibody conjugate could also be attached to the noble metal surface of a quartz crystal microbalance.

- After attachment of the dendrimer-antibody conjugate to the surface of the detector, the antibody retains its ability to bind analyte.

- Showed that our piezoelectric immunosensor could be used to detect a bioanalyte.

- Showed that QCM cell design and flow configuration are critical parameters in appropriate interpretation of data obtained with such devices.

- Demonstrated that a generation five PAMAM dendrimer with hydroxy surface groups also appears to adsorb to gold surfaces. The ability of PAMAM dendrimers to adsorb to noble metal surfaces is an interesting result, and may be pertinent to other applications such as protective coatings.

- Dendrimer-antibody conjugates have also been constructed on the solid phase rather than performing the conjugation and purification tasks in solution and then depositing the conjugate onto the solid phase. This approach of conjugate construction may be useful in device production.

- For comparative purposes, we have immobilized antibodies to QCM transducers with PAMAM dendrimers, polyethyleneimine, and protein A. All three schemes yield comparable results (similar frequency drops) when challenged with 1 mg / mL of analyte.

- We initiated development of PAMAM dendrimer-antibody conjugates which potentially can serve as the detection component in a tab immunosandwich assay.

- We determined that PAMAM dendrimers adsorb strongly to a variety of materials such as cellulose, nitrocellulose, paper and nylon. This indicates that PAMAM dendrimers should serve well for anchoring bioreceptors to these solid phase materials.

Results below are relevant to decontamination schemes. The goal of this work was to develop surface modified PAMAM dendrimers that will allow for the more rapid decomposition of chemical warfare agents in neutral pH solutions.

- Developed facile chemistry for the surface modification of PAMAM dendrimer through the controlled reaction with hydrophobic epoxyalkanes.

- Determined the stoichiometry and the chemical structure of hydrophobe substitution which will still allow the dendrimer to maintain water solubility.

- Completely characterized these products by a range of analytical techniques (^{13}C and ^{1}H NMR, ESI-MS, HPLC and SEC).

4. List of Publications

H.M. Brothers II, L.T. Piehler, D.A. Tomalia, "Slab-gel and capillary electrophoretic characterization of dendritic polymers", J. Chromatogr. A, Vol. 814, 233-246 (1998).

D.A. Tomalia, H.M. Brothers II, "Regiospecific conjugation to dendritic polymers to produce nanodevices", IBC Library Series Publication #1927, Biological Molecules in Nanotechnology: The Convergence of Biotechnology, Polymer Chemistry and Materials Science, 107-119 (1998).

R. Yin, Y. Zhu, D.A. Tomalia, "Architectural copolymers: rod-shaped, cylindrical dendrimers", J. Am. Chem. Soc., Vol. 120, 2678-2679 (1998).

D.A. Tomalia, "Dendrimers: nanoscopic modules for the construction of higher ordered complexity" in Modular Chemistry, Proceedings of the NATO Advanced Research Workship on Modular Chemistry, NATO ASI Series, 183-191, J. Michl(editorr), Kluwer Academic Publishers, The Netherlands (1997).

M.K. Lothian-Tomalia, D.M. Hedstrand, D.A. Tomalia, A.B. Padias, H.K. Hall, Jr., "A contemporary survey of covalent connectivity and complexity. The divergent synthesis of poly(thioether) dendrimers. Amplified, genealogically directed synthesis leading to the de Gennes dense packed state", Tetrahedron, 53(45), 15495-15513 (1997).

D.A. Tomalia, P.R. Dvornic, S. Uppuluri, D.R. Swanson, L. Balogh, "Skeletal macromolecular isomerism: a comparison of dendritic polymer properties to those of classical macromolecular architectures", Polym. Mater. Sci. and Eng., 77, 95-96 (1997).

Y. Sayed-Sweet, D.M. Hedstrand, R. Spindler, D.A. Tomalia, "Hydrophobically modified poly(amidoamine)(PAMAM) dendrimers: Their properties at the air-water interface and use as nanoscopic container molecules", J. Mater. Chem., 7(7), 1199-1205 (1997).

D.A. Tomalia, R. Esfand, "Dendrons, dendrimers and dendrigrafts", Chemistry & Industry, 11, 416-420 (2 June 1997).

P.R. Dvornic, D.A. Tomalia, "Recent advances in dendritic polymers", Current Opinion in Colloid & Interface Science, 1, 221-235 (1996).

5. Participating Scientific Personnel

The following scientists were supported in part during a portion of the grant period from October 1995 to December 1998. No advanced degrees were earned by any of the participants during the time period of the grant.

Dr. Herbert M. Brothers II
Dr. Lars Piehler
Dr. Ralph Spindler
Dr. Donald A. Tomalia (no cost to the grant)
Dr. Bing Wang
Mr. Quinhua Weng

6. Technology Transfer

a. We continue to transfer conjugation technology, improvements in conjugation technology, and conjugate samples to Drs. H.D. Durst and R. Yin at the ERDEC where testing and evaluation are performed. Indications are that dendrimer –

antibody conjugates are improving the performance of ALERT tickets used in the detection of potential biological warfare agents. In addition, Drs. Durst and Yin are investigating the potential use of dendrimer – antibody conjugates in other devices and instruments which are of interest to the Army.

b. Patent No. 5,714,166, Bioactive and/or Targeted Dendrimer Conjugates (Tomalia, et. al) was issued this past year. This patent covers dendritic polymers which are conjugated to a variety of materials including biological response modifiers and/or target directors.

c. The names and organizations of those in the government to whom we regularly send reports and papers are discussed in (d) below.

d. During the course of the year, Dr. Donald Tomalia has had numerous telephone discussions and email exchanges with Dr. H.D. Durst of ERDEC. Dr. Tomalia also has had numerous telephone and email exchanges with Dr. Gary Hagnauer of the ARL, who is co-director (along with Dr. Tomalia) of the ARL-MMI Dendritic Polymer Center of Excellence.

e. Improved processes in dendrimer conjugation chemistry in conjunction with developmental work being conducted at the Army (Drs. H.D. Durst and R. Yin) may allow manufacture of more sensitive single-use biodetection devices.

f. See attached slides for technology highlights.

g. The ARL Center of Excellence for the Study of Dendritic Polymers in Army Applications is continuing in full force at MMI. Dr. Gary Hagnauer is the key contact at ARL and we continue to collaborate with ARL scientists including Drs. McKnight, Beck Tan, Wood, Miller, Stenhouse, Trevino and Tassinari. We also continue to work with Dr. Eric Amis' group at NIST and Dr. Tomalia has had joint publications with Dr. Amis this past year.

h. Dr. Rui Yin of Michigan Molecular Institute is currently working at ERDEC and is the key vector for introducing dendrimer and dendrimer conjugation technology into the Army laboratories. Dr. Yin will probably join the ARL within the next year.

i. Dendritech, Inc. supplied us with PAMAM dendrimers and additional financial aid at no cost to the project. In addition, a visiting scientist, Dr. Teruyo Sugiura, spent some time this past year investigating the possibility of stabilizing enzymes with dendritic polymers. Funding for these studies was through a local company at no cost to the grant. We studied interactions with alpha-chymotrypsin and horseradish peroxidase as model enzymes. The enzymes did retain catalytic activity, even when covalently coupled to dendrimers, but our studies exhibited no strong evidence of stabilization of these particular enzymes when mixed or conjugated to PAMAM dendrimers. There was some evidence that the horseradish peroxidase was more thermally stable than the unconjugated enzyme when it was conjugated to a PAMAM dendrimer. Further studies should be done

with enzymes that are relative to the interests of the Army since different enzymes will probably behave differently owing to differences in the tertiary structures and surface properties of the proteins.

7. Honors and Awards Received

D.A. Tomalia appointed to a Distinguished Visiting Professorship at Columbia University, New York, New York (1998 to date).

D.A. Tomalia appointed as the Scientific Director at the Center for Biologic Nanotechnology, U. of Michigan Medical School, Ann Arbor, Michigan (1998 to date).

D.A. Tomalia appointed as the Vice President of Technology, Director of MMI Nanocenter, and Chief Scientist at Michigan Molecular Institute, Midland, Michigan (1998 to date).

8. Inventions

No invention disclosures were made as part of this program during this last year.

9. Bibliography

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2. D. A. Tomalia and H. D. Durst, Topics in Current Chemistry, Springer-Verlag, Berlin Heidelberg, *165*, 193 (1993) and references therein.

3. Singh, P.; Moll, F. III; Lin, S.; Ferzli, C.; Yu, K.S.; Koski, K.; Saul, R. G.; Cronin, P. Starburst™ dendrimers: enhance performance and flexibility for immunoassays. Clin. Chem. 1994, 40, 1845-1849.

4. Singh, P.; Moll, F. III; Lin, S.; Yu, K.; Ferzli, C.; Saul, R.; Diamond, S. Starburst™ dendrimers: characterization, derivatization and bio-conjugation. PMSE 1993, 70, 237-238.

5. Singh, P.; Moll, F. III; Lin, S.; Ferzli, C. Starburst™ dendrimers: a novel matrix for multifunctional reagents in immunoassays. Clin. Chem. 1996, 42, 1567-1569.

6. H.M. Brothers II, L.T. Piehler, R. Spindler and D.A. Tomalia, "Dendritic Polymers: A New Class of Polymers for Decontamination and Detection of Chemical and

Biological Warfare Agents", interim progress report for DAAH04-95-1-0652, ARO, 1996.

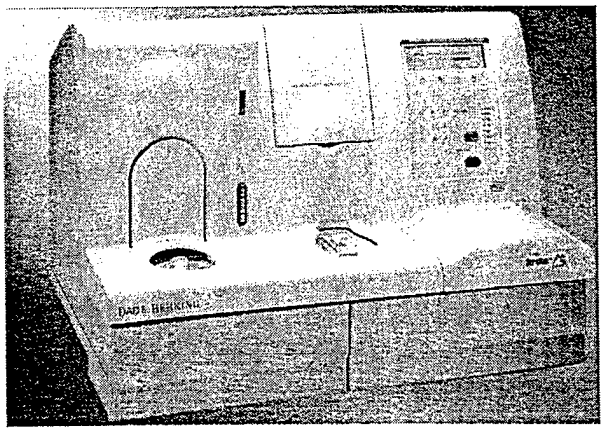
7. H.M. Brothers II, R.Spindler, D.A. Tomalia, "Dendritic Polymers: A New Class of Polymers for Decontamination and Detection of Chemical and Biological Warfare Agents", interim progress report for DAAH04-95-1-0652, ARO 1997.

APPENDIX

(FIGURES)

Dade Behring's STRATUS® CS Instrument

•Commercial Launch in 1998



- Utilizes Dendrimer Technology
- Combines Speed of Homogenous Assay with Partitioning Advantages of Heterogeneous Assays
- Diagnosis of Myocardial Infarction (heart attack)

Figure 1.

Schematic of Dade-Behring Assay

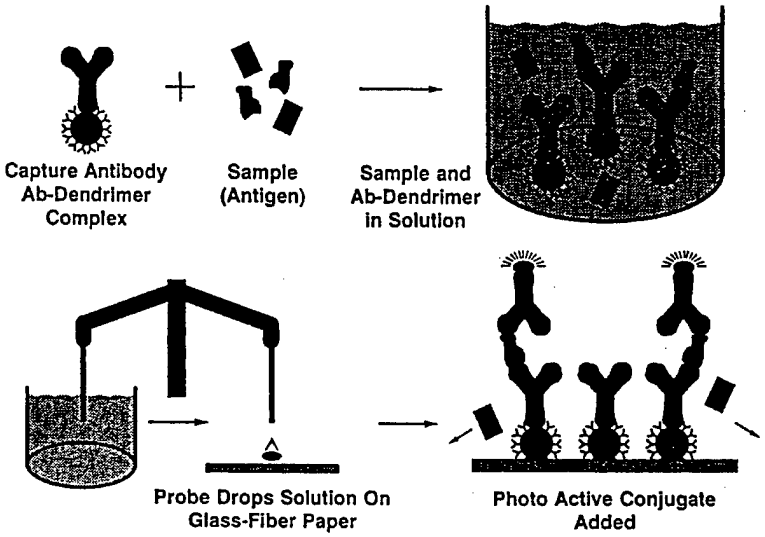


Figure 2.

SMCC / LC-SPDP Chemistry

Macromolecule Conjugation

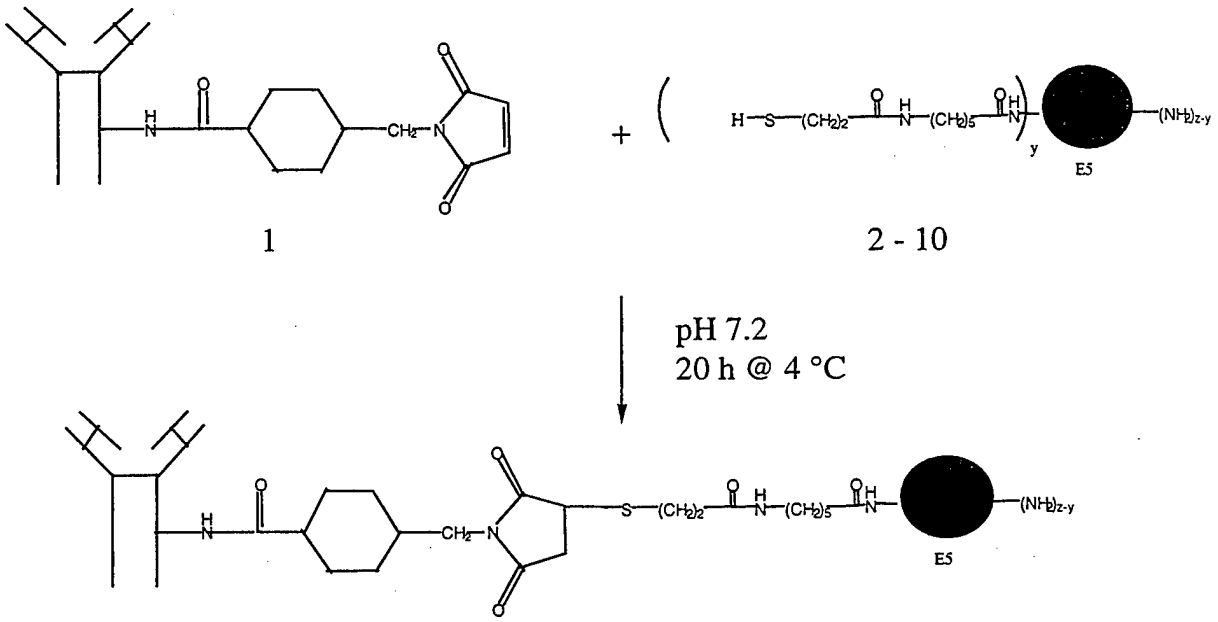
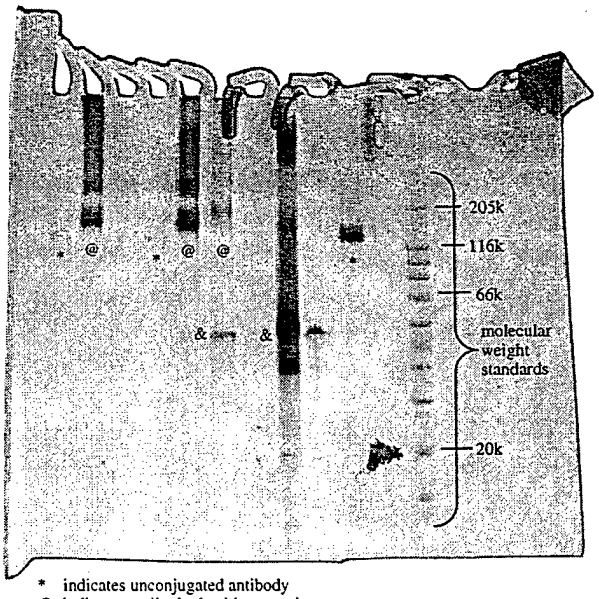


Figure 3.



* indicates unconjugated antibody
@ indicates antibody dendrimer conjugates
& indicates unconjugated dendrimer

Note the difference in migration distances between unconjugated antibody(*) and the dendrimer-antibody conjugates(@). The conjugates clearly have shorter migration distances than the unconjugated antibody thereby indicating that the dendrimer-antibody conjugates have a larger molecular weight than the unconjugated antibody.

Figure 4.

Figure 5.

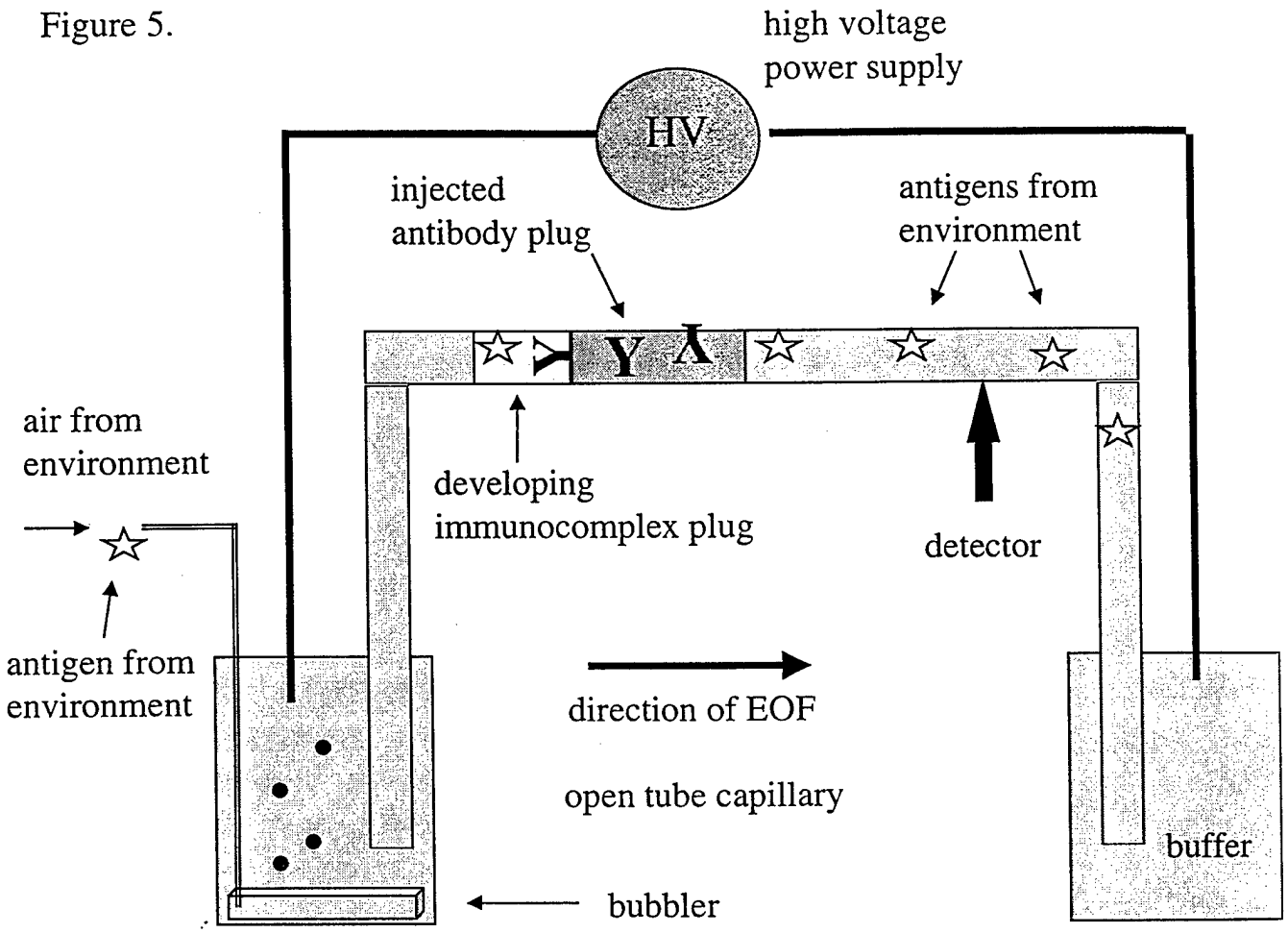

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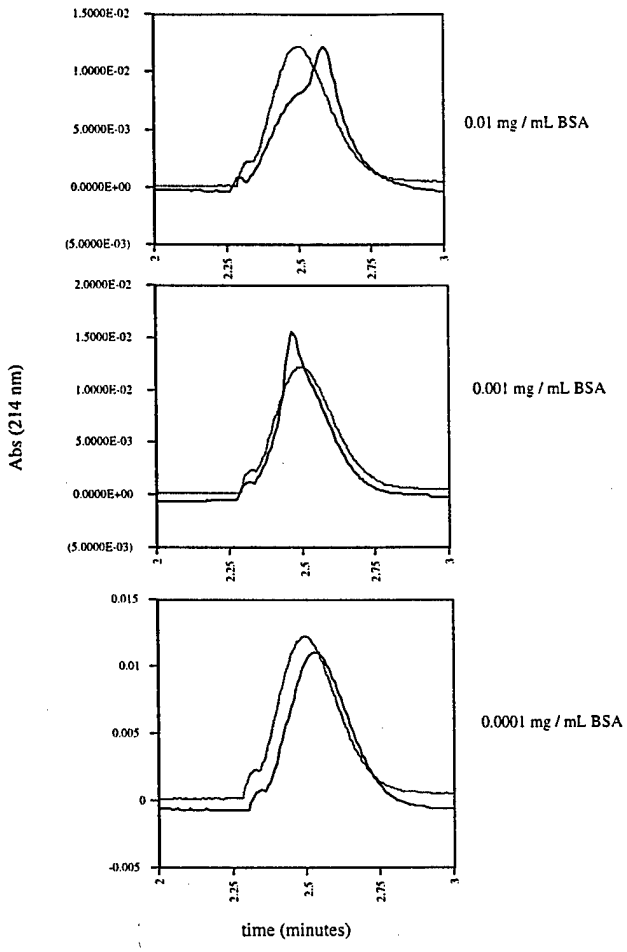
Figure 6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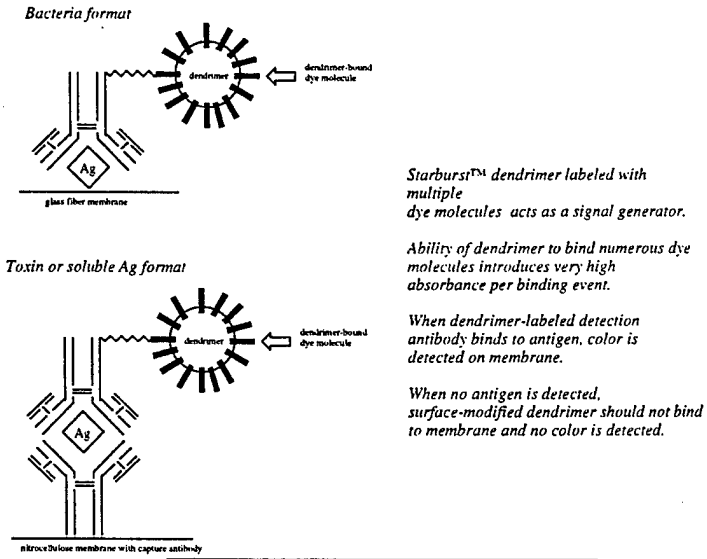

Figure 7. Representation of Immunoassay with Dendrimer-Modified Detection Antibody