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(71) Applicant: **NANOSYNTHONS LLC** [US/US]; 1200 N. Francher Avenue, Mt. Pleasant, MI 48858 (US).

(72) Inventors: **TOMALIA, Donald, A.**; 463 W. Chippewa River Road, Midland, MI 48640 (US). **HEDSTRAND, David, M.**; 506 W. Chippewa River Road, Midland, MI 48640 (US). **NIXON, Linda, S.**; 1225 S. Loomis Road, Mt. Pleasant, MI 48858 (US).

(74) Agent: **KIMBLE, Karen, L.**; Technology Law, PLLC, 3595 N. Sunset Way, Sanford, MI 48657 (US).

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[Continued on next page]

(54) Title: PYRROLIDONE DERIVATIVES, OLIGOMERS AND POLYMERS

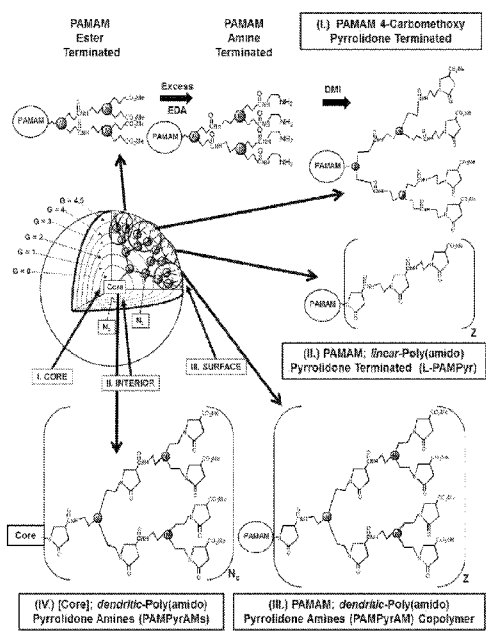

Figure 10

(57) Abstract: Simple organic structures, organic/inorganic polymers, and other substrates have been made, all of which have at least one pyrrolidone moiety present, and found to exhibit low toxicity, low complement activation features and may be used to reduce protein interactions with drug conjugates while enhancing *in vivo* residency times for these conjugates when used as an injectable composition; thus these compounds can be used as substitutes for PEG in PEGaylation. Surprisingly, these compounds also exhibit unique intrinsic fluorescence (IF) or non-traditional fluorescence (NTF) properties that currently cannot be explained by traditional photochemistry and fluorescence paradigms are described. These compounds have a variety of applications such as in cellular imaging, gene transfection, bio-diagnostics, biosensing, fluorescence directed surgical resections, drug delivery, forensics, environmental diagnostics, mineral/gemstone characterization, counterfeit goods detection, tracer studies related to liquid/water flow, oil field enhancements and diagnostics, prevention of photo-bleaching, and LED display enhancements and others.

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PYRROLIDONE DERIVATIVES, OLIGOMERS AND POLYMERS

BACKGROUND OF THE INVENTION

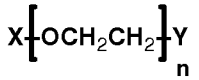
Field of the Invention

This invention generally concerns the use of derivatives/analogues of poly(vinylpyrrolidone) (PVP) , namely poly(pyrrolidone) macrocyclics, oligomers and low molecular weight polymers as masking agents for biological materials in a manner analogous to those obtained using poly(ethyleneglycol) (PEG). Additionally, the small molecule pyrrolidone intermediates (*i.e.*, the monomeric precursors) as well the new poly(pyrrolidone) oligomers/polymers obtained from these derivatives exhibit unique intrinsic fluorescence (IF) or non-traditional fluorescence (NTF) properties that cannot be explained by traditional photochemistry and fluorescence paradigms. These compounds, oligomers and polymers have a variety of applications such as in masking drugs for biological applications, cellular imaging, gene transfection, biosensing, fluorescence directed surgical resections, drug delivery, forensics, mineral/gemstone characterization, oil field enhancement and diagnostics, counterfeit goods detection, tracer studies related to liquid/water flow, fluorescent whitening agents and LED display enhancements and others.

Description of Related Art

PEGylation

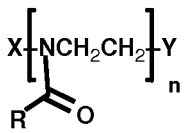
The general concept of PEGylation was first introduced and patented in the 1970's by F. Davis *et al.,* (US Patent 4,179,337 (**1979**)) as a strategy to reduce toxicity, immunogenicity and proteolytic degradation of therapeutic drugs/proteins, while enhancing blood circulation times, drug solubility and PK/PD's of the therapy. The strategy involves the covalent conjugation of poly(ethylene glycol) oligomers (*i.e.*, DP= 4-50) to the desired therapeutic drug or substrate. This PEGylation strategy has provided the basis for launching over a dozen important drugs that include: for cancer treatment such as Doxil/Caelyx® by Ortho/Schering-Plough (**2001**), multiple sclerosis, such as Plegridy® by Biogen (**2014**), cancer related drug Movantik® by AstraZeneca (**2014**), anemia such as Peginesatide® by Affmax Takeda Pharma, (**2012**), to mention a few. This strategy involves the covalent conjugation of low molecular weight PEG oligomers (*i.e.,* < 50-70 KDa) using so-called, "activated PEG reagents" as described below:



where: X and Y can be independently reactive or non-reactive with functionality possessed by the desired protein, polynucleotide or therapeutic drug to be modified; n is from 4-50.

POXylation

Due to a number of shortcomings related to PEG oligomer chemical properties (*i.e.*, oxidative, enzymatic stability or immunogentic problems with chronic use and due to higher MW fractions), (G.T. Hermanson, Chapter 18 in *Bioconjugate Techniques*, Second Ed., **(2008)** 707-742), there has been an active quest for alternative polymer types and compositions. This has led to an early report by Zalipsky *et al.* (see S. Zalipsky, *et al., J. Pharm. Sci.*, **(1996),** *85*, 133-137) describing the usefulness and potential advantages of poly(oxazolines) as a replacement for PEG's. Very recently, work has focused on the use of poly(oxazolines) (F. M. Veronese, *et al., Bioconjugate Chem.*, **(2011)**, *22*, 976-986) and POXylation conjugates of rotigotine (*i.e.*, dopamine agonist) for the treatment of Parkinson's disease by Serina Therapeutics (www.serinatherapeutics.com). As such, these POXylation protocols involve the use of "activated POX reagents" for attachment to these therapeutic drugs/proteins as described below:



where: X and Y can be independently reactive or non-reactive with functionality possessed by the desired protein, polynucleotide or therapeutic drug to be modified; n is from 4-50.

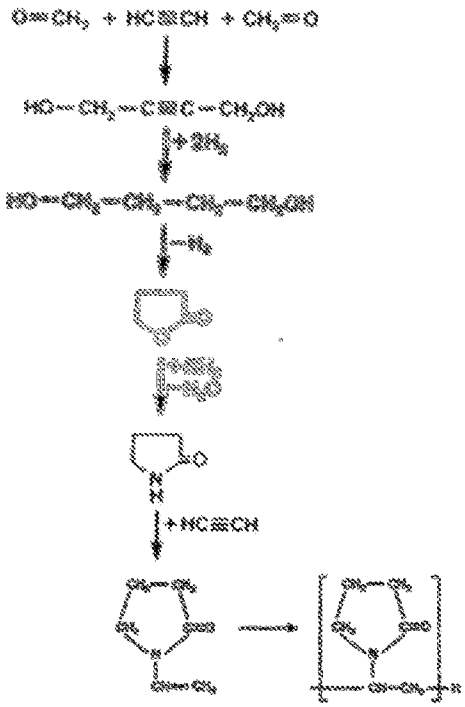
Historical Use of Poly(vinylpyrrolidone) (PVP) as an Injectable Synthetic Polymer in Humans

Historically, the most extensively studied/documented synthetic polymer composition utilized for internal injection in humans has been poly(vinylpyrrolidone) (PVP). For over 75 years, since its discovery in the late 1930's, this polymer has been injected in over 500,000 human patients with virtually no adverse toxicity, immunogenicity or other negative effects for use as a very successful blood substitute/extender (Sultana, *et al., J. Pakistan Med. Association*, (**1978**), 28 (10), 147-153). More extensive and

contemporary human use of this synthetic polymer composition as an injectable has been hampered solely by concerns that higher molecular weight polymer fractions (*i.e.*, >70 KDa) may not be adequately excreted through the kidney and be accumulated *in vivo* with multiple injections and over extended time. This hypothesis appears to have been confirmed by several well documented medical studies (Wang *et al., J. Cutan Pathol.*, **2006**, 33, 454-457). The specific medical condition created is referred to as; *PVP accumulation disease* or *Dupont-Lachapelle Disease*. This medical condition is widely recognized to be due to non-excretable, higher molecular weight PVP fractions present in currently available poly(pyrrolidone) products. Although this medical condition is not fatal, it is considered to be a negative feature for PVP since higher molecular weight PVP fractions have been proven difficult if not impossible to remove (Pfirrmann *et al.,* US Patent 6,080,397, **2000**) and will require a significant scientific solution or alternative for future use.

Currently, PVP polymers are produced commercially in large quantities by various manufacturers such as BASF. PVP production involves the free radical polymerization of N-vinyl pyrrolidone monomer (N-VP). The –N-VP monomer is obtained by the original Reppe process involving the combination of acetylene, formaldehyde and ammonia under high pressure (see Scheme 1 below).

Scheme 1



The (N-VP) monomer has been successfully polymerized only with free radical catalysts (*i.e.*, peroxides, persulfates) in bulk, solution or in suspension, to give *linear*- PVP with weight- average molar masses ranging from 2500-1,000,000 Da (see Haaf *et al. Polymer Journal*, **1985**, 17(1), 143-152). These linear-PVP polymers are generally obtained as highly poly-dispersed products and were characterized by measuring their viscosities in solution according to a "Fikentscher *K*-value molecular weight relationship" developed by Kern and Cherdron (Kern *et al.*, *Houben Weyl, Methoden der Organische Chemie*, Vol. 14, 4th ed., Georg Thieme Verlag, Stuttgart, **1961**, p.1106), using the relationship between M_w, M_n and K; wherein: $M_w=15K^{2/3}$ and $M_n=24K^2$. The letter K together with an appropriate number is used to relate the molecular weight for the various PVP molecular weight fractions. For example a *K-12* has an average molecular weight of ~ 20,000 Da and *K-90* has a molecular weight of ~ 1M Da. Therefore, PVP with a specified *K*-value and average molecular weight consists of a range of molecular sizes. Based on these viscosity characterization protocols it is clearly apparent that well-defined molecular weight ranges let alone well defined, controllable molecular weights for PVP do not currently exist. Controlling PVP polymer molecular weights, which is related to nanoscale sizes, is a critical issue for many nanomedicine applications, wherein, nanoscale size is known to determine excretion modes, bio-distributions, toxicology and complement activation properties (see for example Kannan *et al., J. Intern. Med.* **2014**, 276, 579-617).

The pyrrolidone moiety as found in PVP (*i.e.*, *Povidone*, trademark of BASF) enjoys an excellent record and universal recognition as a versatile non-toxic, biocompatible, physiologically inert material for a wide variety of medical applications (see Haaf *et al., Polymer Journal*, **1985**,17(1), 143-152). Foremost has been the extensive *in vivo* use of PVP as a blood plasma extender (*e.g.*, Sultana, *et al., J. Pakistan Med. Association*, **1978**, 28, (10), 147-153); wherein, it has undoubtedly saved countless lives. During World War II, (*i.e.*, initiated by I.G. Farben; now Providone by BASF) and in subsequent years (Korean War) [http:/hcvets.com/data/military/korea.htm; page 44], referred to in Sweden as Periston; it is documented that PVP has been used internally *via* injection in over 500,000 human recipients as a blood extender (Sultana, *et al., J. Pakistan Med. Association*, **1978**, 28, (10), 147-153) without any significant evidence of deleterious effects (Ravin *et al., New England J. of Med.*, **1952**, 247, 921-929). Radioactive studies showed that 95-100% of injected PVP (*i.e.,* Periston) was excreted via the urine within 72 hours; 40 % was excreted within 20 minutes; and within 6 hours, virtually all circulating PVP had disappeared from the plasma. Subsequent research has shown that the (PVP) composition exhibits virtually no antigenic

properties (Maurer *et al., J. Immunology*, **1956**, 77(2) 105-110) compared to other synthetic (*i.e.,* polyesters/polyalcohols) or biological polymers (*i.e.*, poly(dextrans) or poly(saccharides)).

Currently, (PVP) is being used as an adjuvant for immobilizing spermatozoa for *in vitro* fertilization protocols (www.coopersurgical.com). Other examples include the use of PVP in applications ranging from cosmetics (*e.g.*, hair sprays) to eye drops and oral pill binding formulations. More recent confirmation of the low cytotoxicity and minimal interaction of the poly(pyrrolidone) moiety with proteins, when presented on the surface of poly(amidoamine) (PAMAM) dendrimers has been reported (Ciolkowski *et al., Nanomedicine, NBM*, **2012**, 8, 815-817; and Janaszewska *et al., Nanomedicine, NBM*, **2013**, 9, 461-464).

As such, there is a critical need to synthesize /control the PVP molecular weight in a range of 3-14 KD (Pfirrmann *et al., U.S. Patent* 6,080,397, **2000**). This is necessary to avoid *in vivo* accumulation of higher MW PVP fractions and be acceptable as an injectable product for *in vivo* applications. Unfortunately, all known polymerization mechanisms (*i.e.*, free radical, anionic, cationic types) for propagating N-vinyl pyrrolidone to produce PVP polymers lead to substantial amounts of uncontrolled, higher molecular weight (*i.e.*, > 14 KDa). PVP products, as well as polymerization side products that make these materials unacceptable for *in vivo* or injectable product applications. More specifically, when PVP containing higher molecular weight fractions (*i.e.*, >14 KDa) are administered intravenously, an *in vivo* accumulation of the polymer may occur which is referred to as "PVP storage disease" or also known as the *Dupont-Lachapelle Disease* (Wang *et al., J. Cutan Pathol.*, **2006**, 33, 454-457). This disease is characterized by symptoms that include dermatosis, rheumatic joint pain, and pulmonary respiratory insufficiency. On the other hand, low molecular weight PVP with a molecular weight of <14 KDa and a K-value less than 17 has been found to be non-allergenic and is quickly removed unchanged by excretion from the blood stream *via* the kidneys. However, all attempts at producing low MW PVP exclusively by free radical polymerization and subsequent ultra-filtration have been unsuccessful (Pfirrmann *et al., U.S. Patent* 6,080,397, **2000**; www.rloginconsulting.com/…pyrrolidone%20backbone%20polymers.pdf).

Fluorescence Discussion

Fluorescence occurs when an orbital electron of an atom, molecule, polymer or nanostructure in the ground state (S_0) is excited to a higher quantum state (S_1) by the

absorption of some form of energy (*i.e.*, usually a photon; hv_{ex}) and then relaxes back to the ground state (see Fig. 1). This two-step process is described as:

1. **Excitation:** $(S_0) + hv_{ex} \rightarrow (S_1)$
2. **Fluorescence (emission):** $(S_1) \rightarrow (S_0) + hv_{em}$ + heat

This relaxation or return [*i.e.*, (S1) → (S0)] to the ground state is accompanied by the emission of lower energy photons of light (hv_{em}), which is referred to as fluorescence (see Fig. 1). (*The Molecular Probes Handbook – A Guide to Fluorescent Probes and Labeling Technologies*, **2010**, 11th Ed.)

A fluorophore in the (S_1) state may return to the ground state (S_0) *via* relaxation pathways involving either radiative emission (*i.e.*, fluorescence), non-radiative events (*i.e.*, heat) or internal intersystem conversion (IC) (*i.e.*, intersystem crossover, (ISC)) to a non-fluorescent triplet excited state (T_1) with time scales on the order of 10^{-10} to 10^{-9} seconds.

These (T_1) species are very sensitive to molecular oxygen and may undergo redox reactions leading to highly reactive superoxide radicals (ROS) and irreversible fluorophore damage referred to as "photobleaching" (Q. Zheng, *et al.*, *Chem. Soc. Rev.*, **2014**, *43*, 1044-1056). These highly reactive oxygen species (ROS) may cause fluorophore degradation or cause phototoxicity by reacting with nearby biomolecules and are in fact pivotal to so-called photodynamic therapies employed in nanomedicine.

Due to vibrational relaxations following excitation, the photon energy emitted from (S_1) will generally be lower than the excitation photon. This results in an increase in the fluorescence emission wavelength which may range from 5-50 nm higher than the excitation wavelength. The difference between the excitation wavelength and the emission wavelength is referred to as the *Stokes shift* (N.J. Turro *et al.*, *Modern Molecular Photochemistry of Organic Molecules,* **2010** University Science Books, *The Molecular Probes Handbook – A Guide to Fluorescent Probes and Labeling Technologies*, **2010**, 11th Ed.).

Fluorescence imaging techniques have broad applications in life sciences and clinical research. However, these techniques critically rely on bright and photostable fluorescent probes. Currently available fluorescent probes for biological imaging mainly include organic fluorophores (Terai and Nagano, *Pflugers Archiv. European J. Physiology*, **2013**, *465*, 347-359) and quantum dots (Chen *et al., Trends Analytical Chemistry*, **2014**, *58*, 120-129). Small organic dyes suffer from several unwanted properties such as poor

solubility, problems with targeting desired cell compartments, rapid irreversible photobleaching, and cell leakage. Inorganic nanoconjugates such as quantum dots are exceptionally bright, photostable, and characterized by narrow emission spectra, but they possess important drawbacks. First of all, they are toxic and that can limit their applications *in vivo*. Moreover, their intracellular delivery raises problems that make it difficult to follow some biological processes (Jamieson *et al.*, *Biomaterials*, **2007**; *28*, 4717-4732).

Fluorescence in the field of biology and nanomedicine has become a major research focus due to their broad applications in cellular imaging, biosensing, fluorescence directed surgical resections (R. Tsien *et al.*, *Proc. of the National Acad. of Sci.*, **2010**, *107*, 4317-4322) and drug delivery (*i.e.*, *theranostics)* (S. Lo, *et al., Mol. Pharmaceutics,* **2013**, *10,* 793-812; R. Tsien *et al., Proc. of the National Acad. of Sci.* **2010,** *107,* 4311-4316).

Applications of fluorescence outside of biology/medicine uses include, but are not limited to: forensics (M.Y. Berezin *et al.*, *Chem. Reviews*, **2010**, 110, 2641-2684); oil field enhancement and diagnostics (M. Amanullah, **2013**, SPE164162); counterfeit goods detection (US 8,735,852, issued May 27, **2014**; Y. Zhang *et al.*, *Dyes Pigm.*, **2008**, *77*, 545); tracer studies related to liquid/water flow (WO 2011/030313, A method for detecting an analyte, *Indian Inst. of Science*, March 17, **2011**); fluorescent whitening agents ; and LED display enhancements (US Pat. Appl. 20140035960, Apple Inc.) and others.

Traditional standard fluorescing agents are usually described as being members of three major categories:

Category I are organic aromatic conjugated polyenes that include small molecules with low molecular weight (<1000 da) and are derived from these conjugated organic aromatics structures.

Category II consists of fluorescent proteins that usually contain one or more of the three key aromatic moieties such as tryptophan, tyrosine and/or phenylalanines.

Category III consists of inorganic nanoparticles derived from cadmium or lead chalcogenides such as heavy metal sulfides or selenides that must have sizes smaller than a Bohr exciton or radius (2-50 nm). Their fluorescence is determined by their size, but do not exhibit the weakness of photobleaching.

The weaknesses of each traditional fluorophore category are as follows: Category I - lack robustness in the presence of oxygen which leads to rapid fluorophore degradation referred to as photobleaching, as well as photo-toxicity resulting from the generation of the

reactive oxygen species (ROS) which may cause cellular damage and potential carcinogenicity; Category II: proteins that may denature, lack robustness in the presence of oxygen which leads to rapid degradation referred to as photobleaching, exhibit immunogenicity; Category III: quantum dots exhibit heavy metal toxicity, blinking fluorescence, lack of solubility for *in vivo* applications, size must be nanometric and precise (2-50 nm).

The pyrrolidone moiety on the other hand which is a critical component of this invention has an excellent record and international recognition as a versatile non-toxic, biocompatible material for a wide variety of medical applications. Foremost has been the extensive use of poly(vinylpyrrolidone) (PVP) as an *in vivo* blood plasma extender (Polyvinyl Pyrrolidone as a Plasma Expander — Studies on Its Excretion, Distribution and Metabolism, Herbert A. Ravin, Arnold M. Seligman, M.D., and Jacob Fine, M.D.). Since the World War II, it has been used in over 500,000 human recipients without any evidence of deleterious effects (H.A. Ravin, *N. Engl. J. Med.*, **1952**, *247*, 921-929).

In another feature, dendritic polymers are known in the art and are discussed extensively in DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y. Dendritic polymers have become recognized as the fourth and most recently reported major class of polymeric architecture (*J. Polym. Science, Part A: Polym. Chem.* **2002**, *40*, 2719-2728).

Three major architecture components of dendrimers, namely the cores, interior compositions as well as their surface chemistries can be readily modified. At the present, dozens of diverse cores, nearly 100 different interior compositions and over 1000 different surface moieties have been reported for dendrimers [*e.g.,* DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y.]. In many cases dendrimer surface modifications have been performed to alter, enhance or obtain new emerging properties such as: to modify/reduce dendrimer toxicity, gain enhanced solubilities, reduce dendrimer-protein interactions/immunogenicity (*i.e.*, dendrimer stealthness), for the attachment of drugs, targeting or imaging agents including traditional fluorophores such as fluoroscein, Rhodamine red or cyanine dyes. Many of these surface chemistry enhanced dendrimer properties have been shown to be invaluable in a variety of life sciences and nanomedicine applications (*e.g.,* US Patent 5,527,524).

As early as 2001, poly(amidoamine) (PAMAM) dendrimers were reported by Tucker *et al.*, (S. Tucker *et al.*, *Applied Spectroscopy*, **2001,** *55*, 679-683) to exhibit intrinsic fluorescence properties that could not be explained mechanistically by any known traditional fluorescence paradigm. This new non-traditional fluorescence (NTF), observed in dendrimers, generally required excitation radiation between 250-400 nm, followed by relaxation to the ground state to produce characteristic emission bands that ranged from the visible to near infrared region (*i.e.*, 400- 750 nm).

Since this early report, the NTF phenomena has been observed in a wide range of different dendrimer families (*i.e.*, interior compositions) all of which appear to have one thing in common, namely they possess multiples of tertiary amines (3^0 –amines) and/or amides in their interior backbone compositions. It is notable that dendrimer terminal/surface functionality did not appear to significantly influence (NTF); however, dendrimer generation level (G. Jayamurugan *et al.*, *Org. Lett.*, **2008**, *10*, 9-12), degree of dendrimer aggregation (P.K. Antharjanam *et al.*, *J. Photochemistry & Photobiology A: Chem.*, **2009**, *203*, 50-55), solvent viscosities (P.K. Antharjanam *et al.*, *J. Photochemistry & Photobiology A: Chem.*, **2009**, *203*, 50-55), low pH's (T. Imae *et al.*, *J. Am. Chem. Soc.*, **2004**, *126*, 13204-13205; L. Pastor-Perez *et al.*, *Macromol Rapid Commun.*, **2007**, *28*, 1404-1409; Y. Wang *et al.*, *J. Nanosci. Nanotechnol.*, **2010**, *10*, 4227-4233; Y. Shen *et al.*, *Chem. Eur. J.*, **2011**, *17*, 5319-5326), aging (D. Wang *et al., J. Colloid & Interface Science*, **2007**, *306*, 222-227), exposure to air or oxidizing reagents (A.J. Bard *et al.*, *J. Am. Chem. Soc.*, **2004**, *126*, 8358-8359; T. Imae *et al.*, *Colloids & Surfaces B: Biointerfaces*, **2011**, *83*, 58-60), and even a few others, did cause enhancements in fluorescence intensities. In addition to the early more ordered, monodispersed dendrimer examples, the (NTF) phenomena was subsequently observed in several other major macromolecular architectures including: (a) random hyperbranched (Y. Chen *et al.*, *Bioconjugate Chem.*, **2011**, *22*, 1162-1170), (b) linear (L. Pastor-Perez *et al.*, *Macromol Rapid Commun.*, **2007**, *28*, 1404-1409) and (c) certain simple branched (S.-W. Kuo *et al.*, *J. of Nanomaterials*, **2012**, 749732, 10 Pages) polymer structures. In spite of many attempts to utilize these unique dendrimer (NTF) properties for imaging biological cells or labeling, the low (NTF) fluorescence emission intensities generally precluded their practical use, except in the presence of certain oxidizing reagents/environment or at low pH's (*i.e.*, 2-3) in order to obtain an adequate emission intensity for certain applications such as gene transfection (Y. Chen *et al.*, *Bioconjugate Chem.*, **2011**, *22*, 1162-1170).

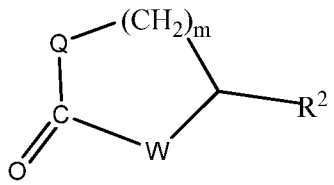
Applying dendrimers – versatile, globular, monodisperse polymers with many surface functional groups – seems to be a solution that may help to overcome limitations of both single organic fluorophores and inorganic nanoprobes. The size of dendrimers places them on the same scale as fluorescent proteins: they are larger than organic dyes and smaller than quantum dots.

Dendrimers have been used as scaffolds for fluorophores. G2 PAMAM dendrimers with PEG chains have been functionalized with two types of fluorophores: carboxy-fluorescein and tetramethyl-rhodamine and tested in Chinese hamster ovary cells (Albertazzi *et al.*, *PloS ONE*, **2011**, *6*, e28450. doi:10.1371/journal.pone.0028450). Higher generations G5 and G6 PAMAM dendrimers have been conjugated with multiple cyanine dyes (Kim *et al.*, *Biophys. J.*, **2013**, *104*, 1566-1575). In many cases covalent attachment of fluorescent labels on the surface of the dendrimer is necessary to evaluate its biological functions *in vitro* or *in vivo*. However, such a modification creates a risk of decreased dendrimer biocompatibility, and affects its biodistribution properties. That is why seeking intrinsically fluorescent dendrimers are of paramount importance.

Clearly, having biocompatible compounds that display fluorescence in the desired wavelength and intensity, with low toxicity, for the intended use has commercial application.

BRIEF SUMMARY OF THE INVENTION

The present invention provides fluorescent cyclic amides, cyclic ureas, cyclic urethanes and cyclic amino amide or amino ureas compounds of the formula



Formula (I)

wherein:

Q is any entity that has a primary amine that reacts to introduce a nitrogen in the ring as shown;

W is N, O, S or $(CH_2)_n$ where n is 0 or 1;

R^2 is -C(O)OH; -C(O)O(C_1-C_4 alkyl); -C(O)-NHR^5 wherein R^5 is C_1-C_4 alkyl or an amido group that can be a moiety on a polymer; and

m is 1-4;

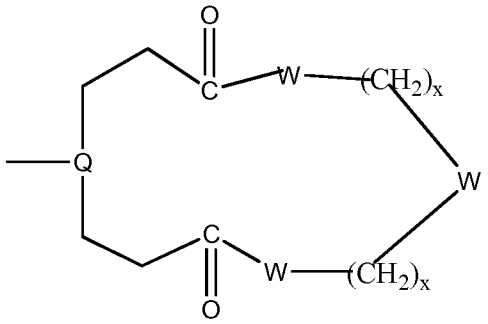
with the proviso that the compound of Formula (I) fluoresces at least 10x the value of its base compound which is QH_2 not having such cyclic amides, urea, urethanes or cyclic amino amide entities present.

The compound of Formula (I) has its emissions are in the visible-near infrared region of 400-850 nm and is non-immunogenic to cells.

Preferred compounds of Formula (I) are those wherein R^2 is -C(O)O(C_1-C_4 alkyl) or -C(O)OH, especially wherein the alkyl is methyl; wherein W is $(CH_2)_n$ where n is 1; and m is 1, especially preferred wherein Q is an amine terminated dendritic polymer or an amine terminated dendron. Some amines of Q are (C_1-C_{20} alkyl) amine, (C_1-C_{20} hydroxylalkyl) amine, (C_1-C_{20} alkyl ether) amines, C_6-C_{14} aryl or (C_6-C_{-14} aryl C_1-C_4 alkyl) amines.

These compounds of Formula (I) are used in a method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by its fluorescence. Thus these compounds can serve as a tracer, biosenser, or imaging agent, prevents photo-bleaching or enhances LED display.

Additional fluorescent macrocyclic amides having from 4 to 14 components of the formula



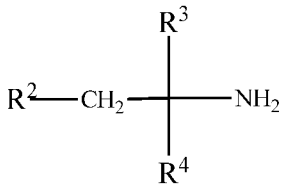
Formula (II)

wherein Q and W are defined as in Formula (I) above; and x is 2 or 3.

These compounds of Formula (II) can be used in a method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by its fluorescence.

Similarly these compounds of Formula (II) can be used as a tracer, biosenser, labeling agent, abiotic sensor or imaging agent; prevent photo-bleaching or enhances LED display.

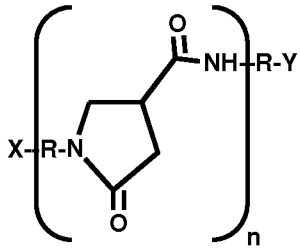
Some primary amines that are suitable Q moieties in Formula (I) are X-Z-N- where X is H, -OH, $-NH_2$, -SH, $-CO_2H$ and Z is (C_1-C_{18} alkyl), (C_6-C_{12})aryl, (C_6-C_{12})arylene, or (C_6-C_{12})alkylarylene, such as (C_1-C_{20} alkyl)amine; (C_1-C_{20} hydroxylalkyl) amine; (C_1-C_{20} alkyl) etheramines; benzylamine, or



where R^2 is $-NH_2$, -OH, or -C(O)OH, and R^3 and R^4 are independently –H or C_1-C_4 alkyl or $–CH_2OH$; a dendrimer, dendron or dendritic polymer having primary amines on its surface; a fatty/lipophilic entity with a primary amine, which when cyclized can form an amphiphilic surfactant that will lead to fluorescent micelles, a protein having a primary amine or any of the common amino acids such as lysine, glycine, tryptophan, tyrosine and/or phenylalanines.

The primary amines that are suitable Q moieties in Formula (1) entity may be presented by (1) small organic molecules, (2) oligomers and polymers which are derived from any of the four major polymer architecture types such as *linear, cross-linked, branched and dendritic polymer types* (DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y.), including both organic and inorganic compositions (i.e., silicas, inorganic oxides, metal chalcogenides, fullerenes, *etc.* or (3) any of the defined Soft or Hard nano-elements (*i.e.*, nanoparticles) as described in (Chapter 8, DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y.).

This invention also concerns activated PYRROLIDONylation reagents made having the Formula (III):



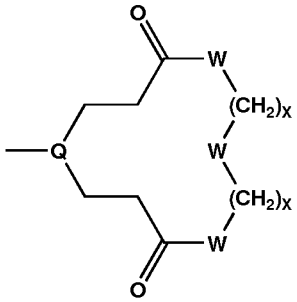
Formula (III)

wherein: R is C_1-C_{18} alkyl, C_6-C_{12} aryl, C_6-C_{12} arylene, (C_1-C_{12} alkyl) C_6-C_{12} arylene; and

X =Y is independently H, -OH, -NH_2, -SH, –CO_2H, alkyne, azido (*i.e.*, click reagents) -or any suitable reactive moiety/ derivative, required for conjugating the PYRROLIDONylation reagents to desired/targeted substrates such as peptides, proteins, antibodies, enzymes, polynucleic acids, drugs, nanoparticles, microparticles, liposomes, micelles, dendrimers, dendrons, dendrimersomes, pharmaceutics, or other biological entities or fragments thereof.

Although not limited to this list, many of the demonstrated small molecule amines are illustrated in Figures 3 A & B. Others that might be mentioned include; (C_1-C_{20} alkyl)amines, (C_1-C_{20} hydroxylalkyl) amine, (C_1-C_{20} alkyl ether)amines, (C_6-C_{18} aryl)amines or (C_7-C_{21} arylalkyl)amines. It is desirable in all cases for these Q entities to possess appropriate conjugation and sequestering (*i.e.*, chelation) functionality that will allow these non-traditional, intrinsic fluorophores (NTIF) structures to be chemically bonded, chelated, associated or adsorbed to a wide range of chemical surfaces , substrates , various macroscopic surfaces such as silica, metals, dental enamel, cotton, wood, food, clothing, glass, and others.

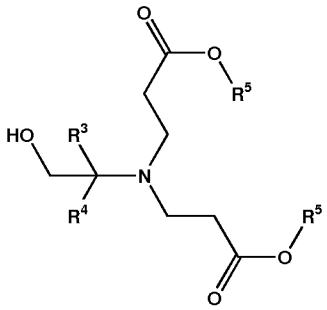
Some of these reactions can form macrocyclic fluorescent amides that are also a part of this invention. The size of the ring can have from 4 to 14 components and still provide the utility described for these compounds of Formula (I) as shown in Table 2. These macrocyclic compounds have the formula:



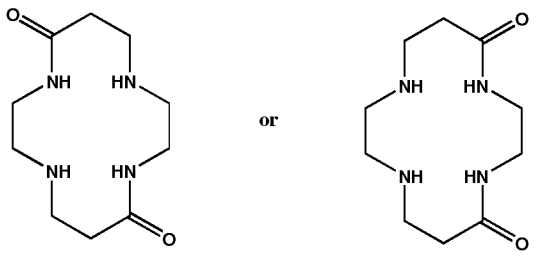
Formula (II)

wherein Q and W are defined as in Formula (I); and x is 2 or 3.

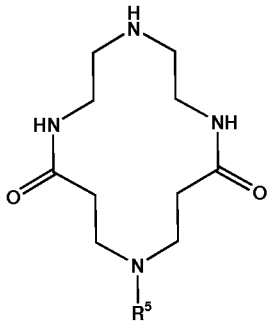
These rings are formed from a general process by reacting a compound of the formula



where R^5 is C_1-C_4 alkyl; R^3 and R^4 are defined as above, with EDA, DETA, TETA, etc. The process provides 7-, 11-, 14-member amide rings. See Fig, 2. While not wishing to be bound by theory, it is believed that 14-member rings form during the process in Examples 18 and 23 having the possible formulas of



and of the formula



for Examples 5, 15 and 29.

When EDA is reacted with 2 equivalents of dimethyl itaconate a dimer is formed that fluoresces. But when 1 equivalent of dimethyl itaconate is reacted with EDA, then a polymer that fluoresces is formed. Thus depending on the conditions, the size of the resulting fluorophore can be controlled.

Thus macrocyclic amides and polymers with amide groups in various rings can be made and used depending of the properties for use, the intensity of the fluorescence desired and the emission wave length from blue to near infared obtained.

These compounds of Formula (I) are used as tracers in medicine for cellular imaging, biosensing, fluorescence directed surgical resections and drug delivery. Applications of fluorescence outside of biology/medicine uses include, but are not limited to: forensics; mineral/gemstone characterization; oil field enhancement and diagnostics, counterfeit goods detection; tracer studies related to liquid/water flow; prevention of photo-bleaching; and LED display enhancements. Thus this invention provides a method of tracing moieties and fluids, and monitoring location in systems for such detection by fluorescence using a compound of Formula (I).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the excitation of a compound in its ground state with energy to an elevated state and relaxes by fluorescence emission back to the ground state.

Figure 2 shows the schematic process for preparing terminated heterocyclic dendrimers.

Figures 3A – 3D show the chemical structure of the compounds of the Examples numbered in Table 2.

Figure 4 shows the chemical structures of the Comparative Examples lettered in Table 2.

Figure 5 graphically shows the data of cellular uptake of G4-PAMAM-pyrrolidone dendrimer at a concentration of 100 μM by mHippoE-18 (blue rhombus), BRL-3A (red circles) and B-14 (green triangles) cells after incubation for 5, 15, 30 minutes, 1, 1.5, 2, 3, 4, 5, 6, 24, and 48 hours.

Figure 6 shows the confocal images of B14, BRL-3A and mHippoE-18 cells treated with 100 μM of PAMAM-pyrrolidone dendrimer for 24h. (**A**) Intrinsic dendrimer fluorescence of unwashed and non-fixed cells. (**B**) Following dendrimer accumulation (blue channel), cells were rinsed once with PBS and stained to visualize cell nucleus (red channel) and plasma membrane (green channel). Before imaging, cells were fixed with formaldehyde.

Figure 7 graphically shows the data for cellular efflux of G4-PAMAM-pyrrolidone dendrimer in a concentration of 100 μM by mHippoE-18 (blue rhombus), BRL-3A (red circles) and B-14 (green triangles) cells after 5, 15, 30 minutes, 1, 1.5, 2, 3, 4, 5, 24, 48 hours.

Figure 8 illustrates the structure for random PAMAM PyrAm megamers.

Figure 9 illustrates the structure of a dendrimer coated with a shell reagent having pyrrolidone moieties.

Figure 10 illustrates the comparison between PAMA dendrimers and the pyrrolidones of this invention.

DETAILED DESCRIPTION OF THE INVENTION

It is understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting. As used in this specification, the singular forms “a”, “an”, and “the” include plural referents unless the content clearly indicates otherwise. The following terms in the Glossary as used in this application are to be defined as stated below and for these terms, the singular includes the plural.

Various headings are present to aid the reader, but are not the exclusive location of all aspects of that referenced subject matter and are not to be construed as limiting the location of such discussion.

Also, certain US patents and PCT published applications have been incorporated by reference. However, the text of such patents is only incorporated by reference to the extent that no conflict exists between such text and other statements set forth herein. In the event of such conflict, then any such conflicting text in such incorporated by reference US patent or PCT application is specifically not so incorporated in this patent.

Glossary

The following terms as used in this application are to be defined as stated below and for these terms, the singular includes the plural. The bold font is not required to meet this definition but to more easily find the term's meaning in this listing.

AEEA means N-(2-hydroxyethyl)ethylenediamine

AEP means 1-(2-aminoethyl)piperazine

Alkyl means straight and branched chain moieties unless otherwise indicated

BOC means *tert*-butoxycarbonyl

BSA means bovine serum albumin

4-CP-PAMAM means 4-carbomethoxy pyrrolidone terminated PAMAM dendrimers

Da means Daltons

DAB means 1,4-diaminobutane

DABCO means 1,4 diazabicyclo[2.2.2]octane

DBA means dibenzylamine

DBI means dibutyl itaconate

DEA means diethanolamine

Dendritic polymers means the dendritic polymer class of random hyperbranched, dendrigraft, dendron or dendrimer polymers, including core-shell tecto-dendrimers

DETA means diethylenetriamine

DMDTB means dimethyldithiobutyrate

DMEM means Dulbecco's Modified Eagle Medium

DMI means dimethyl itaconate

DNA or **RNA** or **Nucleic Acids** means synthetic or natural, single or double stranded DNA or RNA or PNA (phosphorous nucleic acid) or combinations thereof or aptamers, preferably from 4 to 9000 base pairs or from 500 D to 150 kD

DP means degree of polymerization

EA means ethanolamine

EDA means ethylenediamine

EEM means excitation-emission matrix

equiv. means equivalent(s)

FBS means fetal bovine serum

FT-IR means Fourier Transform Infrared Spectroscopy

FWA means fluorescent whitening agents

G means dendrimer generation, which is indicated by the number of concentric branch cell shells surrounding the core (usually counted sequentially from the core)

g means gram(s)

h means hour(s)

halo means fluoro, chloro, bromo, or iodo atom, ion or radical

HB-PEI means hyperbranched -polyethyleneimine

HEDA means (2-hydroxyethyl)ethylenediamine

HMDA means hexamethylenediamine

IA means itaconic acid

IF means intrinsic fluorescence

IR means infrared spectroscopy

ITA means itaconic acid

ITE means itaconic ester

L means liter(s)

L-PEI means linear-poly(ethyleneimine)

MeOH means methanol

mg means milligram(s)

min means minutes(s)

mL means milliliter(s)

MW means molecular weight

µm means micrometer(s)

nm means nanometer(s)

NTF means non-traditional fluorescence

NTIF means non-traditional, intrinsic fluorescent

N-VP means N-vinyl pyrrolidone monomer

PAMAM means poly(amidoamine), including linear and branched polymers or dendrimers with primary amine terminal groups; Starburst™ (trademark of Dendritic Nanotechnologies, Inc.)

PBS means phosphate buffered saline

PEG means poly(ethyleneglycol)

Percent or **%** means by weight unless stated otherwise

PETIM means poly (propyl ether imine)

PIPZ means piperazine or diethylenediamine

PPI means poly(propyleneimine) dendrimers

PVP means poly(vinylpyrrolidone)

PyrAM means pyrrolidone amines

rpm means rotation per minute, the frequency of agitation in a shaking water bath

RT means ambient temperature or room temperature, about 20-25°C

Stealth means non-immunogenic to cells

TETA means triethylenetetramine

TMS means tetramethylsilane

Tracer means a compound used to track the progress or history of a natural process or presence of a compound, such as a histochemical tracer for the study of the composition of cells and tissues; a flow tracer for any fluid property used to track fluid motion; a dye tracer; or any compound that can be located in the environment where it is used.

TREN means *tris*(2-aminoethyl)amine

TRIS means *tris*(hydroxymethyl)aminomethane

UV-vis means ultraviolet and visible spectroscopy

Discussion

PYRROLIDONylation

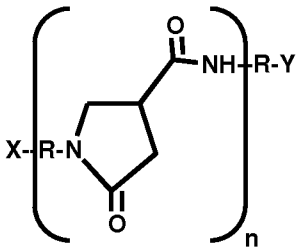
In view of the extensive positive experience of using PVP as an injectable synthetic polymer in humans (*i.e.*, >75 years in >500,000 human subjects) without any known physiological problems except with higher molecular weight fractions (*i.e.*, > 20 KDa; see below), this invention's use of these pyrrolidone compositions are as alternatives to either PEGylation or POxylaton. Both PEGylation and POxylaton have their own deficiencies and unarguably far less *in vivo* documentation in humans than PVP's. Furthermore, an added feature of these new pyrrolidone analogues of PVP over PEGylation and POXylation products is their unexpected intrinsic fluorescence properties. These fluorescence properties have been demonstrated to be invaluable for imaging biologic cells and monitoring *in vivo* transport and biodistribution. Consistent with historical terminology, these new protocols for modifying polynucleotides, peptides, proteins, drugs, and other entities are now referred to as PYRROLIDONylation. A general structural representation of these PYRROLIDONylation reagents is as illustrated below in Formula (III).

These PYRROLIDONylation repeat units may be effectively integrated into any of the four major polymeric architectures including: (a) Linear, (b) Cross-linked, (c) Branched or (d) Dendritic, wherein: the degree of polymerization (DP) = (n) is generally 4-100 and X or Y can be independently reactive or non-reactive with functionality possessed by the desired protein, polypeptide , polynucleotide or therapeutic drug to be modified.

This invention describes such a scientific solution that remediates this widely recognized dilemma by providing well defined, discrete, low molecular weight (*i.e.*, < 100 KDa, preferably <50 KDa or <20 KDa) oligomers/polymeric analogues of PVP that should

be excretable by the kidney and suitable for a wide variety of injectable biomedical applications. As such, these discrete functionalized, low molecular poly(pyrrolidones) also become excellent and viable candidates for use as alternatives or replacements for PEG reagents based on their extensive and largely safe medical use over the past 75 years as injectable blood substitutes/extenders. Furthermore, it has been determined that many of these new small molecule and polymeric pyrrolidone compositions exhibit important unexpected fluorescent properties that are not explicable according to traditional photochemistry paradigm, however, this new fluorescence property has been documented to be very valuable and useful for imaging biological cells and monitoring *in vivo* transport and distribution within an organism.

This present invention now describes novel methods and strategies to produce well defined, low molecular weight poly(pyrrolidone) oligomers possessing highly desirable terminal end group "active drug conjugation functionality sites " that remediate all of the above concerns and shortcomings associated with the use of pyrrolidone compositions for *in vivo* drug delivery applications. In the spirit and context of terminology used for PEG's and POx's , these new protocols and reagents are termed *PYRROLIDONylations*. General examples of these activated PYRROLIDONylation reagent are as described below:



Formula (III)

wherein: R is C_1-C_{18} alkyl, C_6-C_{12} aryl, C_6-C_{12} arylene, (C_1-C_{12} alkyl) C_6-C_{12} arylene; and

X =Y is independently H, -OH, -NH_2, -SH, –CO_2H, alkyne, azido (*i.e.*, click reagents) or any suitable reactive moiety/ derivative, such as those described by G.T. Hermanson, Chapters 1-27 (pp 3-1039) in *Bioconjugate Techniques*, Second Ed., **(2008)**, required for conjugating the PYRROLIDONylation reagents to desired/targeted substrates such as peptides, proteins, polynucleic acids, drugs, nanoparticles, liposomes, dendrimers, pharmaceutics, or other biological entites.

Pharmaceutically acceptable salts and esters of $-CO_2H$ are also included as a compound of Formula (III). These salts and esters are well-known to those persons skilled in drug development and available from the acceptable lists for approved drugs from the FDA for many drugs.

The present invention describes new pyrrolidone polymer compositions (*i.e*, linear, branched, cross-linked and dendritic architectures) , including well defined small molecule pyrrolidone intermediates, with strategies/protocols for their syntheses that permit obtaining unique, well defined pyrrolidone polymer compositions that allow molecular weight (MW) control, especially as oligomers or low molecular weight polymeric compositions. This can be understood by Figure 10.

Presently, such oligomeric/low MW polymeric pyrrolidone compositions are not available by any known polymerization protocols (Pfirrmann *et al.,* US Patent 6,080,397, **2000**; www.rloginconsulting.com/...pyrrolidone%20backbone%20polymers.pdf). The novel, controlled low MW pyrrolidone polymers that constitute the present invention provide dramatically enhanced alternatives to traditional PEGylation (*i.e.,* poly(ethyleneglycol) polymers as discussed above. The PEG polymers possess certain negative property features (*i.e.*, oxidative instability, immunogenic properties) among others. More recently, POXylation (*i.e.*, poly(oxazoline) (J. Milton Harris *et al.*, US Patent 8,088,884, (Jan. 3, **2012**) protocols. These PEGylation and POxylation protocols involve covalent attachment or association of poly(ethyleneglycols) or polyoxzaolines with various proteins, enzymes, drugs or imaging agents in a wide range of *in vivo* drug delivery and imaging applications. The conjugation of biopharmaceuticals to PEG by PEGylation protocols has led to clinical/marketing success of a number of significant, commercially important macromolecular drugs for the treatment of hepatitis C, neutropenia and anemia (F. M. Veronese, A. Mro, G. Pausut, **(2009)** *"Protein PEGylation, Basic Science and Biological Applications. PEGylated protein, drugs; basic science and clinical applications, milestones in drug therapy series"* F.M. Veronese, Ed., pp 11-31, Birkhauser Verlag, Berlin).

In the context and spirit of these earlier technologies, this new poly(pyrrolidone) based technology described by the present invention is named as (PYRROLIDONylation) (*i.e.*, poly(pyrrolidone) protocols. These linear- poly(amidopyrrolidone) (PAMPyr) products are analogues to PEGs (Davis *et al., Adv. Drug Delivery Reviews*, **2002,** 54(4), 457-458) and PEOx (Harris *et al.,* US Patent 7,943,141, **2011**) type polymers. As such they

exhibit low toxicity, low complement activation features and may be used to reduce protein interactions with drug conjugates while enhancing *in vi*vo residency times for these conjugates when used as injectables. These new poly(pyrrolidone) compositions have exhibited amazingly low toxicity [Ciolkowski *et al., Nanomedicine, NBM*, (**2012**), *8*, 815-817; Janaszewska *et al., Nanomedicine, NBM*, (**2013**), *9*, 461-464] and low complement activation (*i.e.,* low immunogenic properties) with *in vivo* stealth properties [N. Spyropoulos-Antonakkakis *et al.*, *Nanoscale Research Letters, (***2015**); 10:210) exceeding those properties recognized for traditional PEGylation protocols. Unexpectedly, many of these new, small molecule pyrrolidone intermediates and their resulting poly(pyrrolidone) (PVP) compositions were found to exhibit new, non-traditional "intrinsically fluorescence" properties suitable for *in vivo* imaging of biological cells or tracking physiological movement and biodistributions in organisms and animal models.

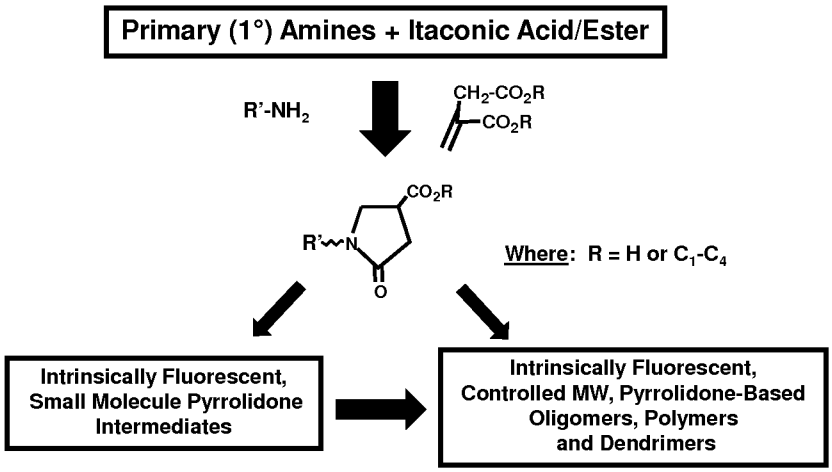
Very recently it has been reported that pyrrolidone terminated PAMAM dendrimers are truly unique compared to all other nanoscale particles due to their very low toxicity (Ciolkowski *et al., Nanomedicine, NBM*, **2012**, 8, 815-817; Janaszewska *et al. Nanomedicine, NBM*, **2013**, 9, 461-464), negligible complement activation properties (N. Spyropoulos-Antonakkakis *et al., Nanoscale Research Letters,* **(2015);** 10:210) and quite remarkably the unprecedented ability to selectively target human atheromatous carotid tissue (*i.e.*, cardiovascular plaque) (N. Spyropoulos-Antonakkakis *et al.*, *Nanoscale Research Letters, (***2015**); 10:210).

Process Discussion

Methods /Processes for Synthesizing Intrinsically Fluorescent, Small Molecule Pyrrolidone Intermediates , Oligomers and Polymers as Alternate Replacements for PEGylation Reagents

These small pyrrolidone molecules are readily prepared by a simple process involving the reaction of primary amines with itaconic acid or its derivatives (*i.e.,*esters, amides, anhydrides) . This general primary amine + itaconic acid derivative reaction scheme may be used to control the MW of these pyrrolidone intermediates as well as the MW's of desired low molecular weight pyrrolidone polymers, all of which exhibit unexpected intrinsic fluorescence (IF) suitable for *in vivo* applications. These strategies are illustrated with the following flow diagram (Scheme 2).

Scheme 2



In this scheme, R is H or C_1-C_4 alkyl; R' is C1-C18 alkyl.

The synthetic strategies used to synthesize heterocyclic/macrocyclic (*i.e.*, pyrrolidone) rings for this invention generally involves the reaction of mono- and poly-1° amines with itaconic acid (ITA), itaconic esters (ITE) such as DMI, or itaconic anhydride. *In all cases the stoichiometry between various 1° amine moieties and the itaconic acid reagents was very critical.* These stoichiometries could be systematically adjusted to produce a wide diversity of 4-carboxylic acid /ester pyrrolidone categories. These categories included: (1) simple molecular structures, (2) bicyclic/ oligomeric /macrocyclic structures or (3) polymeric pyrrolidone product types.

These high yield reactions between simple small molecule primary amines and itaconic acid derivatives were very readily extended to all polymeric architectures bearing primary amines, including dendrimers as well as linear polymers *i.e.*, poly(vinylamines) and poly(allyamines).

The first reaction to form an IF small molecule pyrrolidone intermediate shown by (A) in Scheme 2 requires at least one primary amine such that a monoamine, diamine, triamine molecule can be R, then that amine is reacted with itaconic acid (ITA) or itaconic ester (ITE). Any primary monoamine can be used such as 2-aminoethanol, aminoethylethanolamine (AEEA), *tris*-hydroxylmethyl amine (TRIS), glucosamine, glycine, dodecylamine, or other desired monoamine. The stoichiometry is:

[1° Amine moiety: ITA/ITE]

[1:1] → 2-R(X) Substituted-4-Carboxy Pyrrolidones

[2:1] → 2-R(X)-Substituted-4-Carboxyamido Pyrrolidones

The resulting product has IF and is (A) in Scheme 2.

When a diamine, having 2 primary amines, reacts with ITA or ITE then two pyrrolidone rings can form per diamine. Examples of these primary diamines are ethylene diamine (EDA), Cystamine (CYS), diethylene triamine (DETA), triethylene tetraamine (TETA), or other desired diamines. The stoichiometry may be:

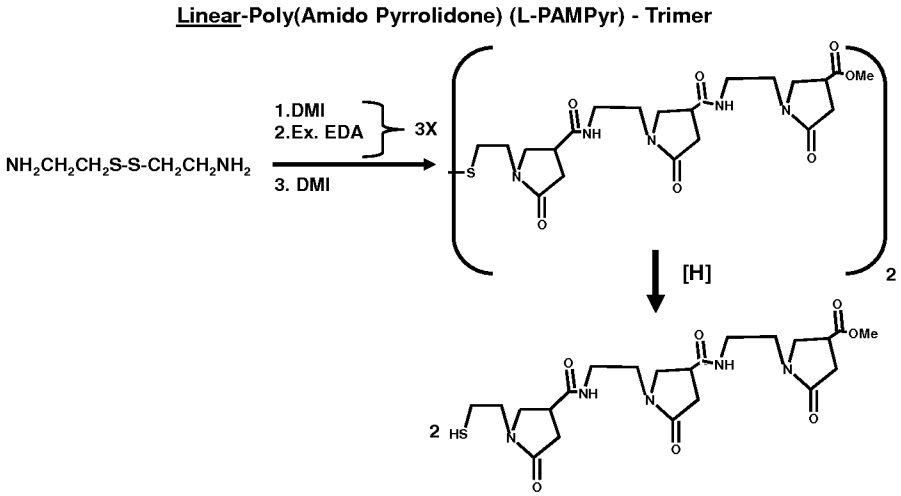
[1° Diamine moiety: ITA/ITE]

[1:1] → R(X) Substituted-4-Carboxy Pyrrolidone Backbone Macrocyclics, Oligomers, Polymers

[4:1] → 2-R(X)-Substituted-4-Carboxyamido Pyrrolidones

[1:2] → 2-[-R(X)-]-Substituted-4- Carboxyamido Di-Pyrrolidones

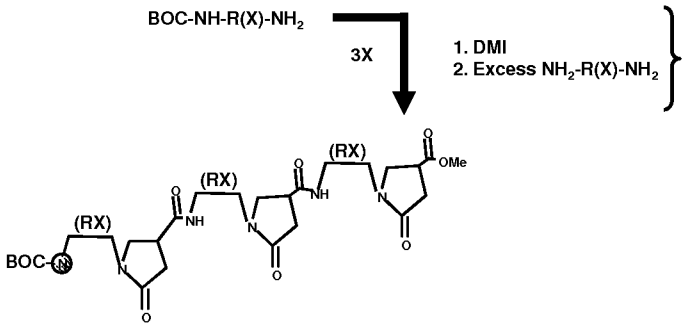
In this latter case, one may perform this [1:2] reaction on cystamine to form the dipyrrolidone intermediate from which one can perform sequential iterations with excess EDA followed with DMI. This iterative sequencing yields important *thiol protected linear*-PAMPyr oligomers of desired lengths that are of high importance for PYRROLIDONylation of various biological substrates as illustrated below:



Alternatively one may perform iterative DMI + excess EDA sequencing on a BOC protected diamine, in which case the number of iterations would determine the length of the PYRROLIDONylation reagent. Of course traditional Merrifield like substrates may also be used for similar protection of such a diamine. That withstanding , appropriate endgroup modifications, cleavage from the resin and or de-protection of the BOC group would provide selective functionalization of these PYRROLIDONylation reagents for suitable conjugation to all desired biological substrates or targets according to procedures described

extensively by G. Hermanson (G.T. Hermanson, in *Bioconjugate Techniques*, Second Ed., **(2008)**.)

Pyrrolidone Oligomers



When a triamine, having 3 primary amines, reacts with ITA or ITE then three pyrrolidone rings may form per triamine. An example of these primary triamines is *tris*(2-aminoethyl) amine (TREN) or other desired triamines. The stoichiometry is:

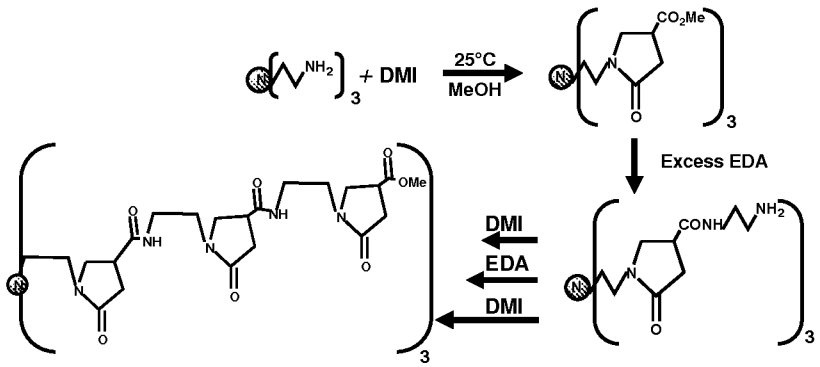
[1° Triamine moiety: ITA/ITE]

[1:1] → Tri- Substituted-[2- R(X), 4-Carboxylic acid/ester Pyrrolidones]

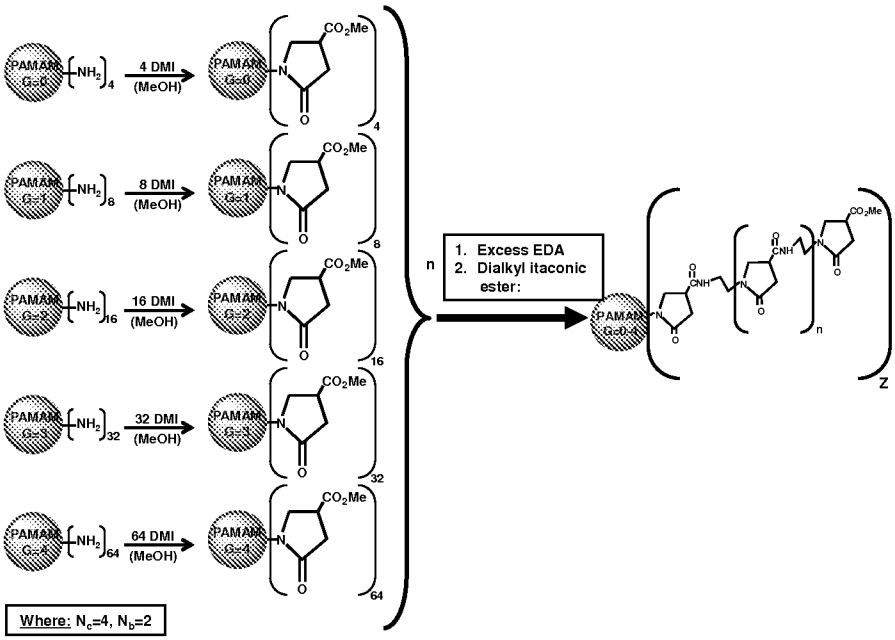
N-[(CH_2-CH_2-NH_2]$_3$ (TREN) + 3 DMI → N-[(CH_2-CH_2-N(4-carboxyl acid/ester) pyrrolidone]$_3$

It should be noted that the first tri-pyrrolidone reaction product formed in this approach may be used as a reactive scaffolding for synthesizing, MW /structure controlled 3-arm branched, star type PYRROLIDONylation reagents. The iteration steps shown below produces the star branched, poly(pyrrolidone) oligomeric product containing nine pyrrolidone rings.

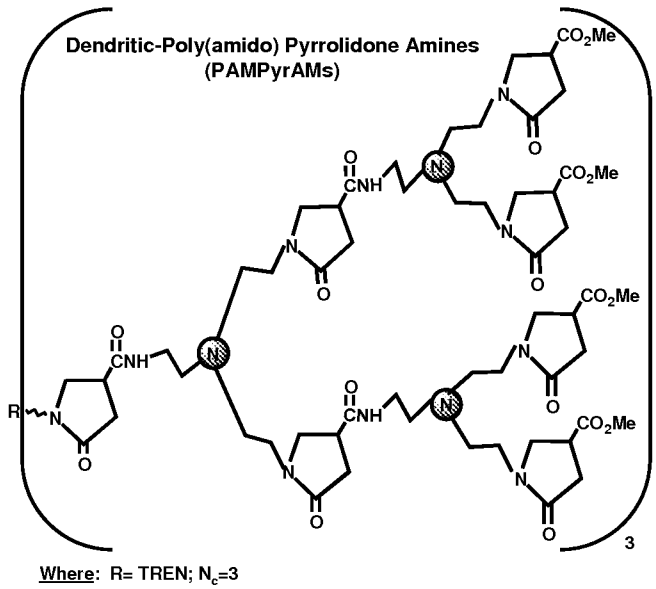
Pyrrolidone-Based Star Branched PAMAM Polymers



By extending this DMI + EDA interation strategy to an amine terminated dendrimer series, one can make the corresponding dendrimer presenting $NcNb^G$ poly(linear) branches as a function of the generation level as illustrated below:



Similarly, one can produce an analogous well defined, tri-dendron type dendrimer structure by merely replacing ethylenediamine (EDA) with tri-(2-aminoethyl) amine (TREN). By using a similar iteration sequence with TREN as above with EDA produces three dendrons around the central N-core of TREN as shown below:



This new unprecedented G=2; tri-dendron dendrimer structure contains exactly 21-pyrrolidone rings and is referred to as a representative member of the poly(amidopyrrolidone amine) **(PAMPyrAM)** family.

[1:2] → Mono(NH_2)-R(X)-Substituted-Di-(4-Carboxyamido Pyrrolidones) → Hyperbranched - R(X)-Substituted-poly-(4-Carboxyamido Pyrrolidone

N-[(CH_2-CH_2-NH_2]$_3$ + 2 DMI → N-[(CH_2-CH_2-NH_2) [(4-carboxylic acid/ester) pyrrolidone]$_2$ → Hyperbranched- Pyrrolidones

[2:1] → Di-(NH_2)-R(X)-Substituted-Mono-(4- Carboxyamido Di-Pyrrolidones)→ Hyperbranched - R(X)-Substituted-poly-(4-Carboxyamido Pyrrolidones)

N-[(CH_2-CH_2-NH_2]$_3$ + 1 DMI → N-[(CH_2-CH_2-NH_2)$_2$ [(4-carboxylic acid/ester) pyrrolidone] → Hyperbranched Pyrrolidones

In Scheme 2, when (B) polymers are formed, they have their MW controlled by this process and are pyrrolidone polymer compositions exhibiting IF, low toxicity and low complement activation properties suitable for *in vivo* biological injection and imaging applications. All polymer contain pyrrolidone moieties in their structure, namely as a surface group(s) or as a part of the structure of the amine that is reacted with ITA, ITC or DMI or both in the structure and on the surface or the polymer. These polymeric compositions can be produced in four different architectural forms, namely; (a) Linear Polymers, (b) Cross-linked Polymers, (c) Branched Polymers, and (d) Dendritic Polymers.

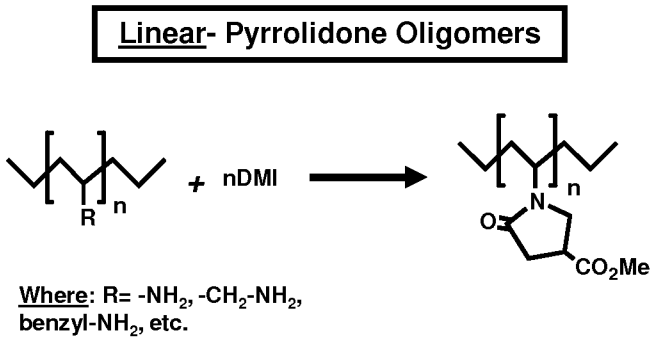
Linear- Pendant Pyrrolidone Oligomers/Polymers:

1°-Polyamines [RX-(NH_2)$_Z$] + ITA or ITE →

In the above reaction some primary polyamines are poly(vinyl amine), poly(allylamine), poly(vinylbenzylamine) or other suitable polyamines having 1 or more primary amines; and Z=degree of polymerization (DP). The stoichiometry is:

[1° Amine moiety: ITA/ITE] = [1:1]

[1:1] → Linear- [Poly-2-(R(X))-Substituted-4-Carboxylic acid/ester Pyrrolidones]$_{DP=n}$



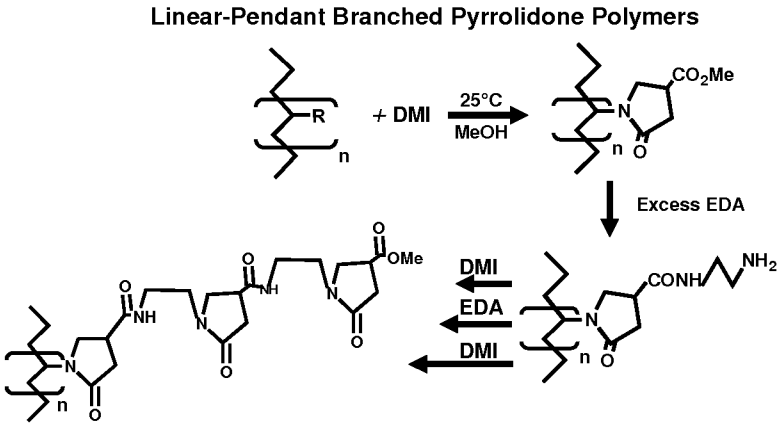
Cross-linked, Linear- Pendant Pyrrolidone Polymers:

[1° Amine moiety: ITA/ITE] = [DP: DP-x]

[DP: DP-x] → Cross-Linked-[2-(R(X))-Substituted Poly-4-Carboxylic acid/ester Pyrrolidones]$_{DP-x}$

One can readily form cross-linked versions of the above linear-pendant pyrrolidone oligomers/polymers (shown above) by simply adjusting the ratio of DMI to primary amine so that the DMI:primary amine stoichiometry is <1:1.

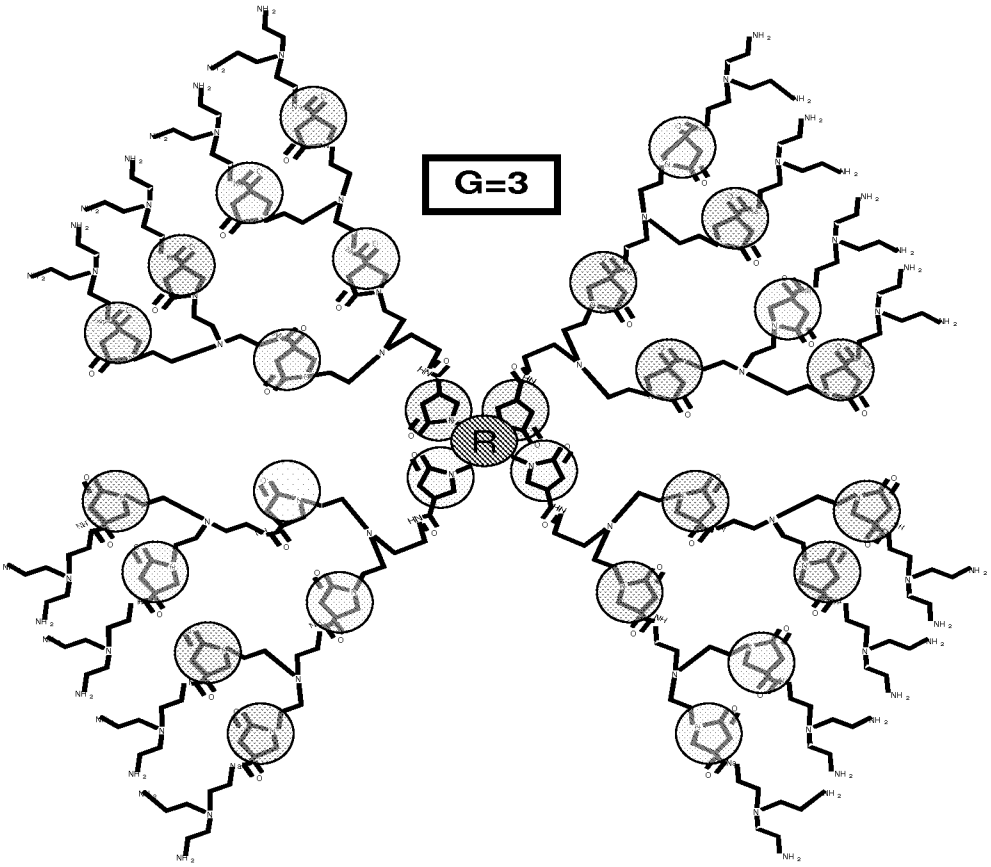
Linear-Pendant Branched Pyrrolidone Polymers



Dendritic Core-Dendritic Surface Poly(Pyrrolidone) Copolymeric Dendrimers:

As shown above , one may grow pure, all poly(pyrrolidone) based dendrons by performing iterative reaction sequences using [1:1] DMI stoichiometry followed by using

excess TREN starting from simple, small molecule primary amine cores such as tris-(2-aminoethyl) amine (i.e.,TREN), ethylene diamine (EDA), 1,4-diaminobutane (DAB), etc. Such a *dendri*-{poly(amidopyrrolidone) amine}-$(NH_2)_Z$; G=3; (PAMPyrAM) dendrimer derived from a core = R= [DAB]; where: N_c=4 , contains exactly 28- pyrrolidone rings and is as illustrated below:



Alternatively, one may grow *dendri*- poly(pyrrolidone) type dendron components from the surface of primary amine terminated dendrimers derived from any desired interior dendrimer composition to produce new, unprecedented [*dendri*-1]-[dendri-2 (pyrrolidone)] type copolymeric dendrimers. This strategy assures complete mathematical control over the total number of pyrrolidone rings that may be introduced as a function of generation and may be predicted according to the traditional expression ; $Z=N_cN_b^G$ were Z=the number of pyrrolidone rings introduced per generation. Therefore total control over the number of pyrrolidone rings desired in a particular PYRROLIDONylation reagent structure may be designed and engineered according to the below mathematics.

One the most important aspects of this invention is the ability to engineer and control molecular weights of poly(pyrrolidone) oligomers, polymers, dendrons and dendrimers. Applying well documented iterative principles /processes developed for dendron/dendrimer based systems it has been possible to synthesize at last three new poly(pyrrolidone) compositions that may be designed and engineered according to mathematically driven principles /expressions as described below and illustrated in Figure 10.

(I). Traditional *Dendri*-PAMAM- 4-Carbomethoxy Pyrrolidone Terminated Dendrimers

Traditional PAMAM dendrimer; # of terminal primary amines = $Z = N_c N_b^G$

Total # of terminal pyrrolidone moieties = $\mathbf{P = Z = N_c N_b^G}$

(II). *Dendri*-PAMAM: *linear*-Poly(amidopyrrolidone) Terminated; (L-PAMPyr) Copolymer; (linear chain modified surface; chain length = n)

Total # of pyrrolidone moieties = $\mathbf{P = nZ = nN_c N_b^G}$

(III). *Dendri*-PAMAM: *dendritic*-Poly(amidopyrrolidoneamines);(PAMPyrAM) Copolymer

Assumptions:

# of base PAMAM dendrimer terminal primary amines =$Z = N_c N_b^G$

of **PAMPyrAM)** surface generations defined by G' and N'_b (Single pyrrolidone as above G'= 0)

Total # of surface pyrrolidone moieties = $\mathbf{P_z = Z\ N'^{G'}_b = N_c N_b^G\ N'^{G'}_b}$

Total # of interior + surface pyrrolidone moieties= $\mathbf{P = Z\ (N'^{G'+1}_b - 1)/(N'_b - 1)}$

Total # of interior + surface pyrrolidone moieties = $\mathbf{N_c N_b^G (N'^{G'+1}_b - 1)/(N'_b - 1)}$

(IV). [Core]: *dendritic*-Poly(amidopyrrolidoneamines) (PAMPyrAMs)

Assumptions:

Total pyrrolidone dendrimer (G' and N'_b as above)

Total # of surface pyrrolidone moieties = $\mathbf{P_z = N_c\ N'^{G'}_b}$

Total # of interior + surface pyrrolidone moieties = $\mathbf{P = N_c\ (N'^{G'+1}_b - 1)/(N'_b - 1)}$

1°-Polyamine Terminated Dendrimers (i.e., Where: Dendrimer-$(NH_2)_Z$=[Core; (N_c)];[Interior;(N_b)]; [Terminal Groups;(Z)])

There must be at least 1 primary amine on the surface of the dendrimer as a Z moiety available for the reaction with ITA, ITE or DMI. Some possible dendritic structures are poly(amidoamine) (PAMAM) dendrimers, poly(propyleneimine) (PPI) dendrimers, poly(lysine) dendritic polymer and others [DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y.]; and $Z=N_cN_b^G$). The stoichiometry is:

[1° Amine moiety : ITA/ITE]= [1:1]

[1:1] → [Dendrimer]-[(2-Substituted-4-Carboxylic acid/ester) Pyrrolidones)]$_Z$

where $Z=N_cN_b^G$.

The generation of the dendrimer can be G=0, 1, 2, 3, 4 (e.g. PAMAM) which is reacted with DMI and methanol to form the desired number of pyrrolidone moieties on the surface in accordingly from 1 to the maximum number of amines available on the surface (*i.e.*, 4, 8, 16, 32, 64, respectively). This is generalized by the following:

[(1° Amine moieties)x : (ITA/ITE)y] = [X:Y] as a function of dendrimer generation (G) so that when Core = N_c=4) then (X), (Y) result as shown in **Table 1.**

Table 1

Core (G)	Z	(X)	(Y)
0	4	[1-3]	[3-1]
1	8	[1-7]	[7-1]
2	16	[1-15]	[15-1]
3	32	[1-32]	[32-1]
4	64	[1-63]	[63-1]
5	128	[1-127]	[127-1]

Megamers:

Random-Structured Dendrimer PyrAm megamers:

A wide range of Random-Structured Dendrimer Pyrrolidone Amine (PyrAm) megamers are readily produced by adjusting the stoichiometries of the 1° amine component **(X)** and the (ITA or ITE) component **(Y)** on any amine terminated dendrimer. More specifically, adjusting these **(X)** and **(Y)** ratios as a function of PAMAM dendrimer generation (G) as shown above in Table 1, will lead to the random structured PAMAM-PyrAM megamers. When **Y>X**, the megamer will present a predominance of pyrrolidones moieties on the surface. When **X >Y** the megamers will present a predominance of 1° amine groups. These megamers are shown in Figure 8.

Structurally Organized Megamers: Core-Shell tecto(Dendrimers):

Figure 9 (A) illustrates a core-shell tecto(dendrimer) as an organized megamer; (B) illustrates a highly reactive megamer surface where a pyrrolidone moiety is present in the shell reagent.

The present invention relates to new unexpected properties observed for certain categories of 4-15-membered amido, urea, urethane and amidoamino heterocycle molecules as well their use in terminated dendritic, functionalized linear or crosslinked polymers that are valuable and useful for a wide range of medical and commercial applications. The molecules are useful as tracers (defined above) having strong non-traditional intrinsic fluorescence.

Non-traditional, intrinsic fluorescent (NTIF) materials have generally been recognized to be polymeric materials containing high multiplicities of tertiary amines. They initially were considered to be derived exclusively from dendrimer structures; however, it is now known that the NTIF phenomenon has been observed in all four major architectural categories.

These NTIF phenomena have never been observed or reported in low molecular weight organic structures such as traditional fluorophores such as those described in traditional fluorophores (Category I above). The present invention concerns classes of small molecule organic materials not possessing any of the attributes or features of traditional fluorescent materials, yet exhibit fluorescence emissions between 300 and 800 nm. One very important distinguishing property is that they generally contain non-aromatic, low molecular weight structures (basic units <500 da) and are characterized by

low toxicity, non-immunogenic constituents that appear to be robust against photobleaching. Furthermore, they may be used independently as individual small NTIF structures or integrated into a broad range of organic/inorganic polymeric lattices or nanoparticles to produce useful fluorescence emission properties or high value and importance in diagnostics, whitening agents, sunscreens, forensics, LEDs, drug delivery, biological cell labeling and imaging. An example of such NTIF nanoparticle is the use of these small structure fluorophores as terminal groups on dendrimers. These NTIF fluorophores may be polymerized, conjugated and/or attached by charge neutralization to substrates varying from nanoscale to macroscale. They are building blocks that show valuable emission fluorescence properties that are enhanced by associating high multiples of these fluorophores on substrates with dimensions ranging from nanometric (nanometer) to macroscale (meters). Their fluorescence properties may be enhanced by associating large multiples of these fluorophores by polymerization, conjugation, aggregation, supramolecular assembly, charge neutralization and physically.

When a wide variety of simple, low molecular weight (*i.e.* <700 Dalton) N-substituted-4-carboalkoxy/carboxylic acid functionalized pyrrolidone compounds (*i.e.*, cyclic amide type heterocyclics), *not even attached to dendrimers or other polymer scaffolding* were tested, they exhibit significant non-traditional fluorescence (NTF) /intrinsic fluorescence (IF) with emissions in the visible-near infrared region (*i.e.*, 400-850 nm). These emissions appear to be influenced by the presence or absence of certain electron withdrawing/donating groups. For example such groups are: hydroxyl, amino, carboxyl, amido, urea, carbamate (i.e., urethane). These simple pyrrolidone derivatives possess various connector functionalities that allow attachment of these NTF fluorophores to a wide range of inorganic/organic particles or scaffoldings including silica, carbon, metals, synthetic polymers, proteins, DNA/RNA, viruses, and others.

Surprisingly, the absorption/emission properties of these simple pyrrolidone based NTIF fluorophores coincide very closely with very desirable features that are required for commercially significant *fluorescent whitening agents (FWAs).* In essence, the objective of an FWA is to use a fluorescent compound that absorbs UV light and converts the energy into visible light of higher wave length (*i.e.*, in the blue region). In this way, a yellow appearance of a substrate can be corrected by the emission of a corresponding amount of blue-violet light by the fluorescent compound. The effectiveness of the fluorescent agent depends on the presence of ultraviolet light in the illuminant.

FWAs are used in many large scale commercial applications such as: textile/fabric/dental whiteners, dental composites, personal care, paints, paper coatings, inks, synthetic polymers, coatings, natural polymers (*i.e.* wool/silk and others), cosmetics, fluorescent tracers for detection of minute leaks (*i.e.*, adhesives/sealants), films, surfaces, fluorescent calibrators for defining nano-porosity in membranes or other substrates or as tracers for drug delivery applications (*i.e.* siRNA, DNA, oncology, other biomedical roles, *etc.*). By far, the greatest use of FWAs is in detergents, and almost every commercial detergent contains one or more FWAs, in proportions of 0.05%-0.3% by weight.

These present NTIF - FWAs are expected to exhibit enhanced properties in the areas of: lower cytotoxicity, lower phototoxicity, biodegradability, non-immunogenicity, retention or rejection to substrates, quenching, shelf stability and be cost competitive with many current traditional UV absorbers and fluorophores; yet present a wide range of very tunable chemistry moieties suitable for applications in many life science/medical applications such as for their use in gene transfection and other drug delivery roles (*i.e.* biocompatible tracers).

PYRROLIDONylation

Based on the extensive and highly positive history for the *in vivo* use of PVP in over 500,000 human recipients during the past 70 years, there has been a very active interest in remediating the shortcomings of this polymeric composition as a replacement for PEG's. [F. M.Veronese *et al.,* J. *Bioactive Compatible Polym.*, (**1995**), *10*, 103-12], and *Macromolecular Chem. Phys*, (**1999**), *195*, 9-79]. These efforts have been directed mainly at avoiding high MW (*i.e.*, >50KDa) PVP fractions by using chain transfer polymerization protocols [Torchillin *et al.*, *Biomaterials*, (**2001**), *22*, 3035-3044]. Unfortunately, the products obtained were very poly-dispersed and generally lacked suitable functionality for covalent attachment to the therapeutic drugs. In another more recent effort, Pfister *et al.*, [US Patent 6,080,397 (**2000**)] attempted to remove high molecular weight fractions by ultra-filtration of commercial PVP, however, without complete success. These failures to remediate these widely recognized deficiencies of commercial PVP were highlighted recently by Login *et al.,* (www.rloginconsulting.com/…pyrrolidone%20backbone%20polymers.pdf) with the suggestion that condensation polymerization strategies be considered for introduction of the highly desirable pyrrolidone ring into the polymer main chain *versus* the use of free radical

polymerization approaches which yield products with the pyrrolidone ring pendant to the main chain.

General Procedure for the Synthesis of N-Substituted-4-Carboxy-alkylate/arylate Pyrrolidones

The appropriate alkyl or aryl amine (0.05 moles) was weighed into a 100 mL round-bottom flask equipped with a magnetic stirrer and containing 15 mL of dry methanol. While stirring, the appropriate dialkyl/diaryl itaconate (0.05 moles /primary amine moiety) dissolved in 15 mL of dry methanol was added dropwise over a period of 10 min. In some cases, a moderate exotherm was noted. The reaction was allowed to stir at RT for 1 h followed by refluxing for 8-10 h. The final product was obtained by removal of the solvent with a Buchi roto-evaporator to give a solid, liquid or syrup with yields ranging from 75-98%. See Table 2 in the Examples and Figs. 3A- 3B for their structures. These final products were characterized by FTIR, ^{1}H-, ^{13}C-NMR. Their UV-vis/fluorescence properties were evaluated with a fluorospectrometer (Spectramax from Molecular Devices) yielding data as described in Table 2 in the Examples.

General Procedure for the Synthesis of N-Substituted-4-Carboxylic Acid Pyrrolidones

Itaconic acid (Sigma-Aldrich) (0.05 moles/primary amine moiety) was weighed into a 100 mL round-bottom flask equipped with a magnetic stirrer. While stirring, the appropriate alkyl or aryl amine (0.05 moles) was added dropwise over a period of 10 min. In some cases, a moderate exotherm was noted with a transformation into a liquid melt, while in other cases each reactant remained as a solid and was intimately mixed. The flask and reaction mixture was surmounted by a reflux condenser and immersed in an oil bath and heated at 125°C for 2-5 h. The formation of water condensate from the reaction can be noticed in the condenser. A 25 mL portion of dry methanol is then added and the reaction mixture is refluxed for 1 h. Removal of solvent on a Buchi roto-evaporator produces the desired pyrrolidone product as an oil, syrup or solid in yields ranging from 59-95%. These final products were characterized by using a Thomas-Hoover capillary melting point apparatus, FTIR and ^{1}H-, ^{13}C-NMR . Their UV-vis/fluorescence properties were evaluated with a fluorescence spectrometer (Spectramax from Molecular Devices) yielding data as described in Table 2.

Functionalized Dendritic Polymers

The first report that carboxylate-terminated PAMAM dendrimers possess weak, but detectable, fluorescence appeared in 2001 (Larson and Tucker, *Applied Spectroscopy,* **2001**, *55*, 679-683). A broad peak with an excitation and emission maximum of 380 and 440 nm, respectively, was observed. Even though the exact nature of the fluorescence was not fully understood, the authors claimed that is was most likely due to an $n \rightarrow \pi^*$ transition from amido groups throughout the dendritic structure. Much stronger fluorescence emission from G2 and G4 amino-terminated PAMAM dendrimers was observed later for very similar excitation and emission wavelengths (Wang and Imae, *J. Am. Chem. Soc.* **2004**, *126*, 13204-13205). Both G2 and G4 PAMAM dendrimers showed a significant pH-dependent fluorescence property. The emission was detectable in acidic conditions for pH lower than 5. Linear relation between fluorescence intensity and dendrimer concentration was observed. Unsuitable low pH value required to observe dendrimer intrinsic blue fluorescence substantially limits its applicability in biological systems.

Later, it has been found that the emission intensity can be dramatically enhanced upon oxidative treatment (Wang *et al., J Colloid Interface Science*, **2007**, *306*, 222-227) probably due to oxidation of tertiary amines. Therefore, G4 PAMAM dendrimer was treated with ammonium persulfate to enhance its intrinsic fluorescence. Such prepared dendrimers were used as carriers of three antisense oligonucleotides enabling gene delivery and bioimaging at the same time (Tsai *et al.*, *Biomacromolecules*, **2011**, *12*, 4283-4290).

More recent confirmation of the low cytotoxicity and minimal interaction of the poly (pyrrolidone) moiety with proteins, as presented on the surface of poly(amidoamine) (PAMAM) dendrimers is reported by Klajnert *et al.* (*Nanomedicine, NBM,* **2012,** *8,* 815-817*; Nanomedicine, NBM,* **2013**, *9,* 461-464). Currently, PVP is being used as an adjuvant for immobilizing spermatozoa for *in vitro* fertilization protocols (www.coopersurgical.com).

The present invention modifies the surface of PAMAM dendrimers (G=0-6) by converting its standard surface chemistries (*i.e.*, ester, amino, hydroxylic, carboxylic) into various heterocyclic or macrocyclic moieties containing; amino, amido, imino, ether, ester, keto, carboxylic or thioether functionalities, but not limited to this list, which has led to dramatic enhancements in the "intrinsic fluorescence" (IF) observed for the functionalized dendrimer. For example, conversion of dendrimer amine terminal groups into 4-

carbomethoxy pyrrolidones as illustrated in Fig. 2 (a) enhanced the intrinsic fluorescence by >18X compared to the amine terminated dendrimer precursor. This dramatically enhanced IF makes it possible to image/label biological cells; whereas, non-heterocylic functionalized dendrimers exhibit very low fluorescence emission intensities that are too weak to use for cell imaging/labeling. Similar results were observed for other heterocylic and macrocyclic moieties as described in Figure 2 (b), (c), (d) and (e).

More specifically, the present invention relates to new, non-traditional fluorescence (NTF), stealth (*i.e.,* non-immunogenic) and enhanced temperature stabilization properties observed for functionalized 2-pyrrolidone, 2-piperidone, 2-aza-cycloheptanone or 2-azetidinone-terminated dendritic polymers. The preferred examples of 4-carboxalkoxy, 4-carboxamido or 4-carboxylic acid derivatives of pyrrolidone are obtained by reacting a precursor primary amine, (*e.g.,* -NH_2)-terminated dendritic polymers with certain functionalized methacrylate ester, methacrylic acid or methacrylamide reagents to produce new and novel dendritic, linear, branched or crosslinked polymers either terminated or functionalized or both with ester, carboxylic acid or amido substituted 2-pyrrolidone moieties. These pyrrolidone terminated dendrimers are made by the process described in WO2004/069878, published 19 August.

Other analogous and suitable small to large (4-15 membered) heterocyclic moieties, such as 2-azetidinone, 2-piperidone, 2-aza-cycloheptanone or macrocyclic amidoamine moieties, may be introduced directly at the surface termini of dendritic or crosslinked polymers or *via* suitable functionality presented by linear or branched polymers using a variety of synthetic methods described in the literature. These heterocyclic functionalities are referred to herein collectively as "idones".

This invention further embodies the reaction of these "idone-terminated/functionalized" polymers with ester, acid or amine reactive reagents to provide new and novel "mixed functionality" dendritic polymeric materials that exhibit and express these new NTF, stealth or enhanced temperature stability properties.

This invention, based on unpublished work at NanoSynthons LLC /The National Dendrimer & Nanotechnology Center as described herein, demonstrates the following unprecedented results:

- A high yield (*i.e.*, 93-98% yield), facile synthetic process (*i.e.*, 1-step) for the conversion of [Diaminobutane-Core]; amine terminated PAMAM dendrimers (*i.e.,*

G=0-6) to 4-carbomethoxy pyrrolidone terminated PAMAM dendrimers (4-CP-PAMAM's) (*i.e.*, G=0-6) has been made and successfully scaled up to multigram quantities.

- A high yield (*i.e.,* 90-98% yield), facile synthetic process (*i.e.,* 1-step) for the conversion of [Cystamine-Core]; amine terminated PAMAM dendrimers (*i.e.*, G=0-6) to 4-carbomethoxy pyrrolidone terminated PAMAM dendrimers (4-CP-PAMAM's) (*i.e.*, G=0-6) has been defined and successfully scaled up to multigram quantities.

- 4-Carbomethoxy pyrrolidone terminated PAMAM dendrimers (*i.e*., G=4.0) have been shown to exhibit >18X greater NTF emission intensity compared to the corresponding amine terminated PAMAM dendrimer (*i.e.,* G=4.0) without the need for oxidizing agents or lower pH adjustments. See Table 2 in the Examples.

- 4-CP-PAMAM dendrimer (*i.e.,* G=4.0) has been demonstrated to enter biological cells by some, as yet to be defined, endocytosis mechanism and exhibited sufficient NTF emission intensity to allow successful imaging of cytoplasmic domains in three different cell lines.(Univ. of Lodz) . See Example I and Fig.6.

- 4-CP-PAMAM dendrimers (*i.e.,* G=0-4) have been shown to exhibit very low cytotoxicity and virtually no "complement activation" features. See Example I.

- Over 30 simple, but different 4- carbomethoxy/carboxylic acid pyrrolidone derivatives have been synthesized, characterized and shown to exhibit significant NTF emission properties having emission maxima ranging from the visible to the near infrared region (*i.e.*, 400 -750 nm). See Table 2 in the Examples. While not wishing to be bound by theory, it is believed that these emission maxima appear to be readily designed and controlled by a combination of selecting suitable excitation wavelengths and appropriate pyrrolidone structure design.

- This NTF phenomena has never been reported for such simple, low molecular weight pyrrolidone structures or functionalized dendrimers. It has the emission intensity needed for many uses that before were not attainable in a non-toxic system.

- These simple, 4- carbomethoxy/carboxylic acid pyrrolidone derivatives, possess suitable chemical functionality to allow them to be conjugated to a wide range of

dendrimers and other polymer architectures for NTF and other cell labeling evaluations.

A method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using a compound of Formula (I) or Formula (II).

Modification of dendrimer surface groups is a commonly applied method to reduce toxicity of dendrimers. Such modification was proposed by Tomalia *et al.* who developed a polyamidoamine (PAMAM) dendrimer with 4-carbomethoxypyrrolidone surface groups (WO 2004/069878, 19 August **2004**). Biocompatibility of this dendrimer has been assessed (Ciolkowski *et al., Nanomedicine NBM,* **2012**, *8*, 815-817; Janaszewska *et al.*, *Nanomedicine NBM,* **2013**, *9*, 461-464). After analyzing the ability of the dendrimer to interact with human serum albumin, its hemolytic activity and toxic effect on mouse neuroblastoma cell line N2a, it was possible to draw a conclusion that PAMAM dendrimer having carbomethoxypyrolidone surface groups reveals superior properties in comparison with unmodified PAMAM dendrimers with amine surface groups (Ciolkowski *et al., NBM,* **2012**, *8*, 815-817).

Further studies on biocompatibility of the modified dendrimer have confirmed their potential towards applicability in nanomedicine. Using three rodent cell lines: Chinese hamster fibroblasts (B14), embryonic mouse hippocampal cells (mHippoE-18) and rat liver derived cells (BRL-3A), it has been shown that the modified dendrimer has not induced cell apoptosis, has not caused reactive oxygen species (ROS) generation and has not changed mitochondrial membrane potential (Janaszewska *et al.*, *Nanomedicine NBM,* **2013**, *9*, 461-464). Generally, for all three cell lines, the dendrimer has been non-toxic. Such a result gives rise to a question whether the dendrimer enters cells. To address the question, the uptake of the modified PAMAM-pyrrolidone dendrimer for the same three rodent cell lines was done. Monitoring whether the dendrimer enters a cell was possible thanks to a unique property of pyrrolidone-modified PAMAM dendrimers: they possess strong intrinsic fluorescence (λ_{exc}=370 nm, $\lambda_{max\ em}$=440 nm).

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the invention.

Materials and Methods Used in the Examples

Materials

All chemical reagents were purchased from commercial suppliers.

Solvents for the synthesis of the dendrimer were purchased from Sigma-Aldrich.

All cell culture reagents were purchased from Gibco® (Germany).

Flasks and multiwell plates for *in vitro* studies were obtained from Nunc (Germany). SensoPlate™ 96-well glass-bottom plates for confocal microscopy were from Greiner Bio-One (USA).

PBS and FBS were purchased from Sigma-Aldrich.

Trypan blue was purchased from Molecular Probes (USA).

NeuroDio and RedDot1 were purchased from Biotium (USA).

Chinese hamster fibroblasts (B14; ATCC no CCL-14) cell line was purchased from ATCC (USA). Embryonic mouse hippocampal cell line (mHippoE-18) was purchased in CEDARLANE Laboratories Limited (Canada). Rat liver-derived cell line (BRL-3A) was purchased from Banca Biologica e Cell Factory (Italy).

Fluorescence data was acquired with a Perkin-Elmer LS50B Luminescence spectrometer. Constant slit widths (Both excitation and emission – 3nm) were used and the concentrations were adjusted to give on scale emission responses. Emission responses were divided by concentration to give response per gram and multiplied by a scaling factor chosen to set the relative response of the standard, fluorescein-Na, to 1,000,000.

Fourier Transform Infared Spectroscopy (FTIR) 1600 from Perkin Elmer

Anasazi 60 MHz NMR

All the products can also be purified using Sephadex LH-20 in methanol. The columns used here have about 400 g dry Sephadex LH-20. The void volume used for the dendrimer

samples is 300 mL. Fractions are taken in test tubes of ~ 18 mL each (20 mL tubes). Under these conditions G=0 comes out initially at ~ fraction 6 or 7 and G=5 at fraction 2 or 3. Fractions are monitored using a silica gel TLC plate. Two lines are drawn on the plate with each line marked with 0.5 cm separation and each fraction is spotted on every other mark. The plate is simply placed in an iodine chamber. Fractions containing product are collected and stripped on a rotary evaporator.

General Synthesis Scheme

The synthesis of pyrrolidone terminated PAMAM dendrimers were made by the method of Tomalia *et al.* (WO2004/069878, published 19 August **2004**). In a similar matter described by WO2004/069878, all dendritic polymers such as dendrons, dendrimers, dendrigrafts, core-shell tecto(dendrimers), hyperbranched polymers with terminal primary amines can be reacted in this manner, including Examples 1-25. In the following Table 2 and Example I, these compounds were made by this method. General Processes and specific examples are numbered. Comparative Examples are lettered. Example I shows the process to make, by the similar method, the compound tested in the utility discussed.

Example 1: General preparation of N-alkyl-4-carbomethoxypyrrolidinones from primary amines

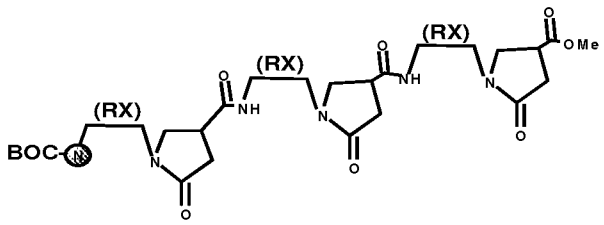
Mono 1° amines (*i.e.,* $-NH_2$) containing a variety of functionalities that exhibit orthogonal reactivity to carboxylic acids may be converted to the corresponding 4-carboxylic acid pyrrolidones in high yield. These products were obtained by heating various stoichiometric quantities of the 1° amine and carboxylic acid in the presence of toluene at ~ 125°C using a Dean-Stark apparatus. After removal of predicted stoichiometric amounts of water (~ 2-3 hrs.), the products are obtained as hygroscopic, powdery solids or brittle glasses exhibiting some level /degree of fluorescence when exposed to UV radiation (265-395 nm). The products were characterized by FTIR, ^{1}H-NMR, ^{13}C- NMR and UV/vis-fluorimetry. (Figure 3C, compounds 26-37, Table 2)

Stoichiometry: **[**1° amine moiety: ITA] = **[1:1]** provides compounds of Formula (I) where W is 1; R^2 is $-CO_2H$; m is 1; and Q is X-R-N; and [1° amine moiety: ITA/ITE] = **[4:1]** provides compounds of Formula (I) where W is 1; R^2 is $-CO_2H$; m is 1 and Q is R(X)N where R is C_2-C_{18} alkylene, $-(NHCH_2CH_2)_n$, arylene, alkylarylene, oligomers, macrocyclics, fused bicyclics, linear poly(amides), n is 0, 1-4; and X is –OH, $-NR_2$, NHR, -SH, $-CO_2H$. These compounds can be further reacted to form polymers containing

pyrrolidone rings in a linear polymer or as a heterocyclic entity containing 2 pyrrolidone moieties. More specifically, the reaction can be described as follows.

To a solution of 50 mmol of primary amine dissolved in 15 mL of MeOH was added to 50 mmol of DMI (7.9g). The solution was stirred at RT overnight, and then the solvent was removed by distillation *in vacuo* to give the desired product. See structures on Figure 3C, compounds numbered 26-37 and data in Table 2.

Subsequent reaction of these intermediates produces the expected *linear*-poly(amido-pyyrolidone) (PAMPyr) macromolecules which may be considered to be analogues to PEG and poly(oxazoline) type oligomers. For example a mono-BOC-$NR(X)NH_2$ initiator unit followed by linear iterative growth with DMI and excess $NH_2R(X)NH_2$ would yield a macromolecule with the general structure shown below after several iterations:



However, the major product type obtained appears to be dependent upon the spacer length of R. When the spacer length R is long, the major product obtained appears to be the *linear*-poly(amido-pyyrolidone) **(PAMPyr)** macromolecules. When the spacer R is appropriate to favor intramolecular ring formation, one obtains either *fused bicyclic pyrrolidone products and/or macrocylic pyrrolidone products* such as shown in Figure 3D, compounds 40 and 41).

Example 2: General preparation of N-alkyl-pyrrolidinone-4-carboxylic acids from primary amines

To 50 mmol of ITA (6.5g) was added 50 mmol of primary amine. The mixture was heated at 125°C for 2 hr. The mixture was triturated with MeOH to give a white precipitate that was isolated by filtration.

Example 3: General preparation of N-alkyl-pyrrolidinone-4-(N-2-aminoethyl)carboxamides from N-alkyl-carbomethoxypyrrolidinones

A solution of N-alkyl-4-carbomethoxypyrrolidinone in MeOH (50% by weight) was added to excess EDA (25 mol per mol of ester) with stirring. The solution was maintained

at RT for 4 days. Excess EDA was removed by distillation, *in vacuo*, followed by azeotropic distillation with toluene, *in vacuo*, to give the desired product.

Example 4: General preparation of N-alkyl-pyrrolidinone-4-(N-2-(*bis*(2-aminoethyl)aminoethyl)carboxamides from N-alkyl-carbomethoxypyrrolidinones

A solution of N-alkyl-4-carbomethoxypyrrolidinone in MeOH (50% by weight) was added to excess TREN (25 mol per mol of ester) with stirring. The solution was maintained at RT for 4 days. Excess TREN was removed by dilution with water and ultrafiltration using a 1kDa cutoff regenerated cellulose membrane to give the desired product.

Note! Using an iterative sequence consisting of reacting a primary amine with DMI to give the 4--(N-2-(*bis*(2-aminoethyl)aminoethyl)carboxamide pyrrolidone ,as above , followed by reaction with an excess of TREN produces a generational sequence for a series of PAMPyrAM dendrons. These dendrons are represented by the Category (IV) ; [Core]; dendritic-poly(amido)pyrrolidone amine dendrons in Figure 10.

Example 5: General preparation of polymeric N-aminoalkyl-pyrrolidinone-4-(N-aminoalkyl)carboxamides from primary diamines

To a solution of 50 mmol of primary diamine (*e.g.* EDA 3.0g) dissolved in 15 mL of MeOH was added 50 mmol of DMI (7.9g). The solution was stirred at RT for 4 days, and then the solvent was removed by distillation *in vacuo* to give the desired product.

The 2, 4-diamino pyrrolidones, derived from the stoichiometric reaction of 2 moles of diamine with 1 mole of itaconic acid, may be used as intermediates in combination with 2, 4-dicarboxylic acid pyrrolidones derived from the reaction of 1x mole amino acid with itanonic acid to produce the corresponding linear-amido-pyrrolidone (*i.e.*, amido-pyrrolidone nylons) which may be considered to be *linear*- poly(amido-pyrrolidone) mimics of poly(ethyleneglycol). (See Figure 3D, compounds 38 and 39)

When stoichiometry of 1° amine moiety: itaconic acid/ ester = 4:1, one obtains the corresponding di-substituted pyrrolidones (i.e., 2-(NH_2-R(X))-4-(CONH-R(X)-NH_2) Pyrrolidones) as described below:

NH_2-R(X)NH_2 + itaconic acid/ester→ 2-(NH_2-R(X))-4-(CONH-R(X)-NH_2) Pyrrolidone

Examples of these 2,4-disubstituted pyrrolidone products are shown in Figure 3D, compounds 42 and 43).

Preparation of PAMAM Dendrimer Pyrrolidinone Surface from Primary Amine Surface Dendrimer and Dimethyl Itaconate

Example 6: [Core:DAB];(4→2); *dendri*-{poly(amidoamine)-(Pyr-4-$CO_2Me)_4$};(G=0); PAMAM Dendrimer; MW = 1049

To a stirred mixture of DMI (1.3 g, 8.2 mmol, 10 % excess) in 5 mL of MeOH cooled to 5°C was added dropwise a solution of: [DAB core];*dendri*-{poly(amidoamine)-$(NH_2)_4$ }; (G = 0); (PAMAM) dendrimer, (1.0 g, 1.8 mmol, 7.3 mmol amino groups) in 5 mL of MeOH over 2 – 3 minutes. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was evacuated free of volatiles using a Buchi rotary evaporator. The resulting residue was dissolved in 10 mL of water and washed with diethyl ether (3x 5 mL). The aqueous layer was stripped free of volatiles and the resulting residue re-dissolved in 10 mL of MeOH. The volatiles were removed from this sample using a Buchi rotary evaporator. This process was repeated three times to give, after a final solvent removal under high vacuum, 1.8 g (96% yield) of the title compound that has the following spectra:

^{13}CNMR (75MHz, D_2O) δ 23.32, 32.00, 33.72, 35.62, 36.22, 41.94, 48.64, 49.55, 52.35, 52.79, 174.55, 175.43, 176.75.

Example 7: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CO_2Me)_8$};(G=1);PAMAM Dendrimer; MW = 2466

To a stirred mixture of DMI (1.9 g, 12 mmol, 10 % excess) in 5 mL of MeOH cooled to 5°C was added a solution of: [DAB core];*dendri*-{poly(amidoamine)-($NH_2)_8$}; (G = 1); (PAMAM) dendrimer, (2.0 g, 1.4 mmol, 11 mmol amino groups) in 5 mL of MeOH dropwise over 2 – 3 minutes. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was evacuated of volatiles using a rotary evaporator and the resulting residue dissolved in 10 mL of water. This mixture was washed with diethyl ether (3x 5 mL). The aqueous layer was stripped of volatiles and the resulting residue re-dissolved in 10 mL of MeOH. The volatiles were removed using a rotary evaporator. This process was repeated three times to give, after a final evacuation with high vacuum, 3.3 g (95% yield) of the title compound that has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 23.32, 32.41, 33.74, 35.66, 36.25, 36.61, 41.96, 48.79, 49.83, 51.08, 52.80, 174.69, 175.42, 176.73.

Example 8: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CO_2Me)_{16}$};(G=2);PAMAM Dendrimer; MW = 5300

To a stirred mixture of DMI (2.5 g, 16 mmol, 10 % excess) in 10 mL of MeOH cooled at 5°C was added dropwise a solution of: [DAB core];*dendri*-{poly(amidoamine)-$(NH_2)_8$};(G = 2); (PAMAM) dendrimer, (3.0 g, 0.91 mmol, 14.6 mmol amino groups) in 15 mL of MeOH over 2 – 3 min. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was evacuated of volatiles using a rotary evaporator and the resulting residue dissolved in 20 mL of water. This mixture was washed with diethyl ether (3x 5 mL). The aqueous layer was stripped of volatiles and the resulting residue redissolved in 10 mL of MeOH. The volatiles were removed using a rotary evaporator. This process was repeated three times to give, after a final evacuation with high vacuum, 4.6 g (95% yield) of the title compound.

In a second purification procedure, the reaction mixture was diluted to 100 mL in MeOH and ultrafiltered to give 8 retentate volumes of permeate using a tangential flow ultrafiltration apparatus containing regenerated cellulose membranes with a 1000 molecular weight cut off . The retentate was stripped of volatiles using a rotary evaporator followed by high vacuum to give 4.6 g (95% yield) of the title compound.

The title compound has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.38, 33.70, 35.59, 36.20, 36.55, 41.92, 48.73, 48.81, 49.52, 51.06, 52.74, 174.38, 174.63, 175.35, 176.68.

Example 9: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CO_2Me)_{32}$};(G=3);PAMAM Dendrimer; MW = 10969

To a stirred mixture of DMI (3.2 g, 20.3 mmol, 10 % excess) in 15 mL of MeOH cooled at 5°C was added: PAMAM dendrimer, DAB core, G = 0 (4.0 g, 0.58 mmol, 18.5 mmol amino groups) in 15 mL of MeOH dropwise over 2 – 3 min. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was evacuated of volatiles using a rotary evaporator and the resulting residue dissolved in 30 mL of water. This mixture was washed with diethyl ether (3x 5 mL). The aqueous layer was stripped of

volatiles using a rotary evaporator followed by high vacuum to give 6.0 g (94% yield) of the title compound.

In a second purification procedure, the reaction mixture was diluted to 100 mL in MeOH and ultra-filtered to give 8x retentate volumes of permeate using a tangential flow ultrafiltration apparatus containing regenerated cellulose membranes with a 1000 molecular weight cut off. The retentate was stripped of volatiles using a rotary evaporator followed by high vacuum to give 6.0 g (94% yield) of the title compound.

The title compound has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 32.43, 32.60, 33.71, 35.62, 36.22, 36.60, 41.95, 48.76, 48.84, 48.94, 49.54, 51.08, 52.77, 174.35, 174.63, 175.33, 176.65.

Example 10: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CO_2Me)_{64}$};(G=4);PAMAM Dendrimer; MW =22,307

To a stirred mixture of DMI (4.7 g, 30 mmol, 10% excess) in 20 mL of MeOH cooled at 5°C was added: PAMAM dendrimer, DAB core, G = 0 (6.0 g, 0.42 mmol, 27 mmol amino groups) in 25 mL of MeOH dropwise over 2-3 min. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was evacuated of volatiles using a rotary evaporator and the resulting residue dissolved in 30 mL of water. This mixture was washed with diethyl ether (3x 5 mL). The aqueous layer was stripped of volatiles to give 8.9 g (95% yield) of the title compound.

In a second purification procedure, the reaction mixture was diluted to 100 mL in MeOH and ultrafiltered to give 8 retentate volumes of permeate using a tangential flow ultrafiltration apparatus containing regenerated cellulose membranes with a 1000 molecular weight cut off. The retentate was stripped of volatiles using a rotary evaporator followed by high vacuum to give 8.9 g (95% yield) of the title compound.

The title compound has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.45, 32.60, 33.71, 35.62, 36.22, 36.60, 41.95, 48.76, 48.84, 48.94, 49.52, 51.08, 52.77, 174.32, 174.60, 175.28, 176.61.

Preparation of [DAB: core];*dendri*-{Poly(amidoamine)-(4-Amidoethylamino Pyrrolidone)z};(G=0-5);PAMAM Dendrimers

Example 11: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)_4$};(G=0); PAMAM Dendrimer

To a 250 mL round bottom flask containing a stir bar was added 57 g of a 80% EDA - 20% MeOH mixture, w/w, 100 equivalents per ester. This mixture was cooled to 8°C and a MeOH solution of PAMAM dendrimer, DAB core, G=0 pyrrolidinone surface (MW=1049) (2.0g, 1.91 mmol, 7.6 mmol ester) was added dropwise over about 1-2 min. This resulting mixture was allowed to warm to RT and stirred for 2 days. An infrared spectrum of this material (evacuate an aliquot with high vacuum) indicated the reaction was about 95% complete as determined from the disappearance of the ester carbonyl group at 1738 cm^{-1}. This mixture was stripped of volatiles on a rotary evaporator. The resulting residue was dissolved in 15 mL of MeOH and 40 mL of toluene was added and mixed to form a homogeneous solution. This mixture was stripped on the rotary evaporator to azeotrope the EDA out of the mixture. This process was repeated six times or until a TLC (silica gel, 10% NH_4OH in MeOH) indicated the absence of EDA by development of the dried TLC plate in an iodine chamber. This mixture was dissolved in MeOH and filtered and stripped of volatiles on a rotary evaporator followed by high vacuum at 50°C for 1 h to give 2.1 g (95%) (MW=1161) that has the following spectra:

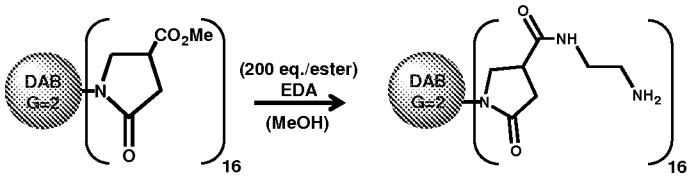
^{13}CNMR (75MHz,D_2O) δ 23.64, 32.32, 34.54, 36.17, 36.61, 39.55, 41.71, 41.92, 48.58, 48.76, 50.28, 52.35, 174.89, 175.45, 176.87.

Example 12: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2))_8$ };(G=1); PAMAM Dendrimer

This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0 Pyrrolidinone amidoethylamine using PAMAM dendrimer, DAB core, G=1Pyrrolidinone methoxy ester (MW=2466)(2g, 0.81 mmol, 6.5 mmol ester), 97g of a 80% EDA- 20% MeOH mixture, w/w, 200 equivalents per ester to give 2.0g (95% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 23.71, 32.29, 32.42, 34.48, 36.14, 36.64, 39.56, 40.15, 41.25, 41.33, 41.91, 48.70, 48.74, 50.22, 50.96, 52.44, 174.52, 174.74, 175.42, 175.80.

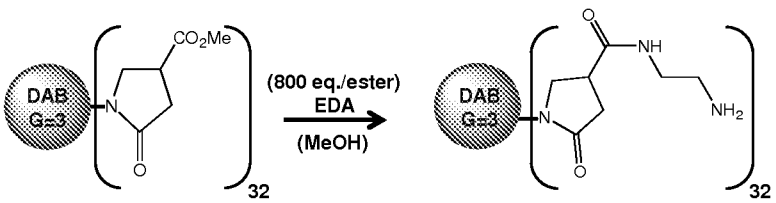
Example 13: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)$)$_{16}$};(G=2); PAMAM Dendrimer



This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0; Pyrrolidinone amidoethylamine using PAMAM Dendrimer, DAB core, G=2 Pyrrolidinone methoxy ester (MW=2466) (2g, 0.81 mmol, 6.5 mmol ester), 97g of a 80% EDA - 20% MeOH mixture, w/w, 200 equivalents per ester to give 2.1g (97% yield) of the title compound that has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 32.51, 32.51, 34.54, 36.20, 36.61, 39.65, 41.54, 41.59, 41.95, 48.82, 48.94, 50.21, 51.08, 174.46, 174.75, 175.42, 175.03.

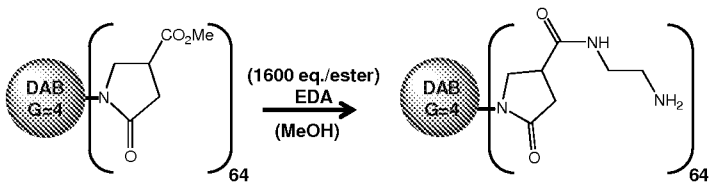
Example 14: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)$)$_{32}$};(G=3); PAMAM Dendrimer



This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0 Pyrrolidinone amidoethylamine using PAMAM Dendrimer, DAB core, G=3 Pyrrolidinone carbomethoxy ester (MW=10969); (3g, 0.27 mmol, 8.7 mmol ester), 522 g of an 80% EDA - 20% MeOH mixture, w/w, 800 equivalents per ester to give 3.0 g (93% yield) of the title compound that has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 32.52, 32.55, 34.55, 36.22, 36.61, 39.66, 41.71, 41.56, 41.95, 48.83, 50.29, 51.11, 174.42, 174.72, 175.41, 175.80.

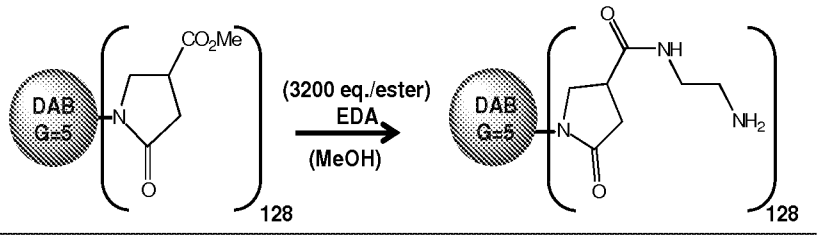
Example 15: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)$)$_{64}$};(G=4); PAMAM Dendrimer



This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0 Pyrrolidinone amidoethylamine using PAMAM Dendrimer, DAB core, G=4 Pyrrolidinone methoxy ester (MW=22307) (2 g, 0.09 mmol, 5.7 mmol ester), 714 g of a 80% EDA - 20% MeOH mixture, w/w, 1600 equivalents per ester to give 2.0 g (92% yield) of the title compound that has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 32.52, 34.55, 36.20, 36.61, 39.70, 41.62, 41.65, 41.95, 48.78, 48.83, 50.29, 51.11, 174.38, 174.69, 175.36, 175.77.

Example 16: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)_{128}$};(G=5); PAMAM Dendrimer

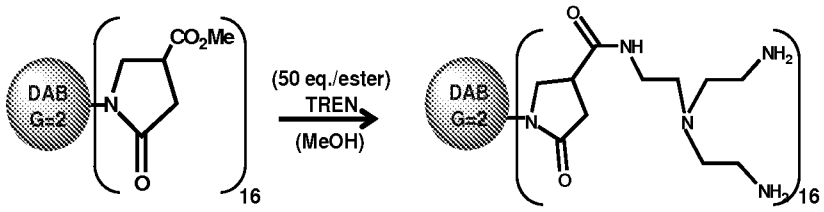


This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0 Pyrrolidinone amidoethylamine using PAMAM Dendrimer, DAB core, G=5 Pyrrolidinone methoxy ester (MW=44782) (1.5 g, 0.03 mmol, 4.2 mmol ester), 1024 g of a 80% EDA - 20% MeOH mixture, w/w, 3200 equivalents per ester to give 1.45 g (91% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.64, 34.55, 36.20, 36.61, 39.73, 41.66, 41.95, 48.76, 48.87, 50.28, 51.16, 174.32, 174.64, 175.30, 176.72.

Preparation of PAMAM Dendrimer, DAB core, Pyrrolidinone-3-Carboamidoethylamino-N,N'-*bis*-ethylamine Surface

Example 17: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-CONH-$TREN)_{16}$};(G=2);PAMAM Dendrimer



This product was prepared in a 100 mL round bottom flask containing a stir bar using PAMAM dendrimer, DAB core, G=2; pyrrolidinone carbomethoxy ester (MW=5300) (1g, 0.19 mmol, 3 mmol ester) dissolved in 3 mL of MeOH that was added to a mixture of TREN (22g, 151 mmol, 50 equivalents per ester) and 6 g of MeOH at 8°C. This mixture was stirred at 25°C for 4 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with MeOH to give a 3-5% solution and purified using a tangential flow ultrafiltration device containing 1K regenerated cellulose membrane for 8-10 retentate recirulations or 2400 – 3000 ml or until a TLC (20% NH_4OH in MeOH) indicated the absence of TREN. The volatiles were removed using a rotary evaporator. The residue was dissolved in MeOH and the resulting mixture was evacuated of volatiles on the rotary evaporator for a total of three times. This residue was evacuated at 40°C for ~ 2 h with a high vacuum to give 8.7g (93% yield) of the desired product (MW=6295) as a white solid. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was redissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 1.2 g (97% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.51, 35.54, 36.22, 36.58, 36.87, 37.71, 41.98, 48.78, 50.24, 51.11, 52.42, 55.47, 174.53, 174.73, 175.07, 175.79.

Preparation of PAMAM Dendrimer Pyrrolidone Sodium Carboxylate Surface

Example 18: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-CO_2^- $Na^+)_{16}$};(G=2);PAMAM Dendrimer

This product was prepared in a 25 mL round bottom flask containing a stir bar using PAMAM Dendrimer, DAB core, G=2 Pyrrolidinone methoxy ester (MW=5300) (1g, 0.19 mmol, 3 mmol ester) dissolved in 3 mL of deionized water that was added to sodium carbonate monohydrate (MW=124) (450 mg, 3.6 mmol, 1.3 equivalents per ester) dissolved in 4 mL of deionized water. This mixture was stirred at 25°C for 3 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with deionized water to give a 3-5%

solution and dialyzed in a regenerated cellulose membrane with a 1000 molecular weight cutoff. The 250 mL dialysate was changed 10 times with 2-18 h between changes. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was re-dissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 1.9 g (97% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.25, 35.15, 36.35, 38.53, 41.91, 48.87, 51.05, 51.20, 174.40, 174.58, 176.77, 180.98.

Preparation of PAMAM Dendrimer Pyrrolidone Amidoethanol Surface

Example 19: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2OH)_4$};(G=0); PAMAM Dendrimer

To a 250 mL round bottom flask containing a stir bar was added 37 g of a 80% EA-20% MeOH mixture, w/w, 100 equivalents per ester. This mixture was cooled to 8°C and a MeOH solution of PAMAM dendrimer, DAB core, G=0 pyrrolidinone surface (MW=2466) (1.5 g, 0.61 mmol, 4.9 mmol ester) was added dropwise over about 1-2 min. This resulting mixture was allowed to warm to RT and stirred for 2 days. An infrared spectrum of this material (evacuate an aliquot with high vacuum) indicated the reaction was about 95% complete as determined from the disappearance of the ester carbonyl group at 1738cm^{-1}. This mixture was stripped of EA using a bulb to bulb distillation apparatus at high vacuum and a pot temperature of 120°C. The bulk of the EA was distilled leaving a viscous residue. This residue was dissolved in MeOH to give a 30% w/w solution and loaded on a Sephadex LH-20 column in MeOH. After a void volume was finished a total of 20 fractions were collected each at 20 mL. Fractions were monitored by spotting on a TLC plate and developing in an iodine chamber. Fractions 6-11 were collected, stripped of volatiles to give 1.44 g (95% yield) (MW=2474) of the title compound that has the following spectra:

^{13}CNMR (75MHz,D_2O) δ 23.64, 32.02, 32.51, 34.55, 36.23, 36.60, 39.55, 41.57, 41.98, 48.84, , 50.33, 51.06, 52.53, 59.91, 174.41, 174.80, 175.44, 175.91.

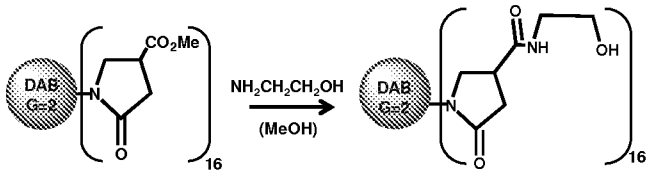
Example 20: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2OH)_8$};(G=1); PAMAM Dendrimer

To a 250 mL round bottom flask containing a stir bar was added 37 g of a 80% EA-20% MeOH mixture, w/w, 100 equivalents per ester. This mixture was cooled to 8°C and a MeOH solution of PAMAM dendrimer, DAB core, G=1 pyrrolidinone surface (MW=2466)

(1.5 g, 0.61 mmol, 4.9 mmol ester) was added dropwise over about 1-2 min. This resulting mixture was allowed to warm to RT and stirred for 2 days. An infrared spectrum of this material (evacuate an aliquot with high vacuum) indicated the reaction was about 95% complete as determined from the disappearance of the ester carbonyl group at 1738cm^{-1}. This mixture was stripped of EA using a bulb to bulb distillation apparatus at high vacuum and a pot temperature of 120°C. The bulk of the EA was distilled leaving a viscous residue. This residue was dissolved in MeOH to give a 30% w/w solution and loaded on a Sephadex LH-20 column in MeOH. After a void volume was finished a total of 20 fractions were collected each at 20 mL. Fractions were monitored by spotting on a TLC plate and developing in an iodine chamber. Fractions 6-11 were collected, stripped of volatiles to give 1.44 g (95 % yield) (MW=2474) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 23.51, 32.19, 32.48, 34.54, 36.23, 36.58, 41.56, 41.93, 48.72, 48.82, 50.32, 51.05, 59.79, 174.40, 174.44, 175.90.

Example 21: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-CONHCH$_2$CH$_2$OH)$_{16}$};(G=2); PAMAM Dendrimer



This product was prepared in a 25 mL round bottom flask containing a stir bar using PAMAM dendrimer, DAB core, G=2 pyrrolidinone methoxy ester (MW=5300) (2g, 0.38 mmol, 6.0 mmol ester) dissolved in 8 mL of MeOH that was added to 5 g of a 80% EA-20% MeOH mixture (w/w), ~10 equivalents amine per ester. This mixture was stirred at 25°C for 3 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with deionized water to give a 3-5% solution and dialyzed in a regenerated cellulose membrane with a 1000 molecular weight cutoff. The 1 liter dialysate was changed 10 times with 2-18 h between changes. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was redissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 2.1 g (97% yield) of the title compound.

Example 22: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-CONHCH$_2$CH$_2$OH)$_{32}$};(G=3); PAMAM Dendrimer

This product was prepared using PAMAM Dendrimer, DAB core, G=3 Pyrrolidinone methoxy ester (MW=10969)(2g, 0.18 mmol, 5.8 mmol ester) dissolved in 8 mL of MeOH and added to 5 g of a 80% EA - 20% MeOH mixture (w/w), 10 equivalents amine per ester. This mixture was stirred at 25°C for 3 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with deionized water to give a 3-5% solution and dialyzed in a regenerated cellulose membrane with a 1000 molecular weight cutoff. The 1 liter dialysate was changed 10 times with 2-18 h between changes. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was redissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 2.1 g (97% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.60, 34.55, 36.23, 36.61, 41.71, 41.59, 41.95, 48.79, 48.84, 48.97, 52.32, 174.43, 174.74, 175.38, 176.85.

Example 23: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2OH)_{64}$;(G=4); PAMAM Dendrimer

This product was prepared using PAMAM Dendrimer, DAB core, G=4 Pyrrolidinone methoxy ester (MW=22,307)(2g, 0.09 mmol, 5.7 mmol ester) dissolved in 8 mL of MeOH and added to 5 g of a 80% EA- 20% MeOH mixture (w/w), 10 equivalents amine per ester. This mixture was stirred at 25°C for 3 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with deionized water to give a 3-5% solution and dialyzed in a regenerated cellulose membrane with a 1000 molecular weight cutoff. The 1 liter dialysate was changed 10 times with 2-18 h between changes. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was redissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 2.1 g (97% yield) of the title compound that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.52, 34.55, 36.23, 36.58, 41.61, 41.95, 48.85, 48.96, 50.30, 59.91, 174.34, 174.40, 174.71, 175.33, 175.80.

Example 24: Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2OH)_{128}$;(G=5); PAMAM Dendrimer

This product was prepared using PAMAM Dendrimer, DAB core, G=5 Pyrrolidinone methoxy ester (MW=44982)(2g, 0.04 mmol, 5.7 mmol ester) dissolved in 8 mL of MeOH and added to 75 g of a 80% EA - 20% MeOH mixture (w/w), 10 equivalents

amine per ester. This mixture was stirred at 25°C for 3 days. An infrared spectrum of this material indicated the complete disappearance of the ester carbonyl frequency at 1735 cm^{-1}. This mixture was diluted with deionized water to give a 3-5% solution and dialyzed in a regenerated cellulose membrane with a 1000 molecular weight cutoff. The 1 liter dialysate was changed 10 times with 2-18 h between changes. The dialyzed mixture was evacuated of volatiles on a rotary evaporator. The residue was redissolved in MeOH followed by removal of volatiles three times and evacuated at high vacuum at 40°C for 1 h to give 2.1 g (97% yield) of the title compound.

Preparation of PAMAM Dendrimer, [DAB core], (G=1) Pyrrolidinone amidoethyl amine pyrrolidinone Surface: Proof of structure for conversion of Pyrrolidone carbomethoxy surface to Pyrrolidinone amidoethylamine surface in a PAMAM dendrimer

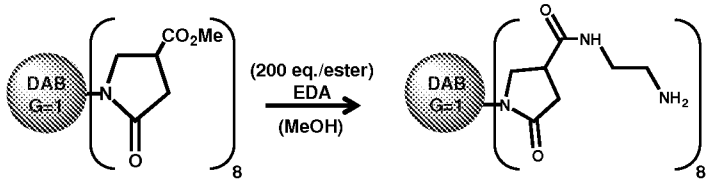
Example 25: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2NH_2)_8$};(G=1); PAMAM Dendrimer

This product was prepared in the same manner as PAMAM Dendrimer, DAB core, G=0 Pyrrolidinone amidoethylamine using PAMAM Dendrimer, DAB core, G=1;Pyrrolidinone methoxy ester (MW=2466) (2g, 0.81 mmol, 6.5 mmol ester), 97g of a 80% EDA- 20% MeOH mixture ,w/w, 200 equivalents per ester to give 98% yield that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 23.71, 32.29, 32.42, 34.48, 36.14, 36.64, 39.56, 40.15, 41.25, 41.33, 41.91, 48.70, 48.74, 50.22, 50.96, 52.44, 174.52, 174.74, 175.42, 175.80.

Example 26: [Core:DAB];(4→2);*dendri*-{poly(amidoamine)-(Pyr-4-$CONHCH_2CH_2$-N-Pyr-4-$COMe)_8$};(G=1); PAMAM Dendrimer

PAMAM Dendrimer, DAB core, G=1 Pyrrolidinone amidoethylpyrrolidinone Surface



To a stirred mixture of DMI (32 mg, 2 mmol, 10 % excess) in 5 mL of MeOH cooled at 5°C was added PAMAM dendrimer, DAB core, G = 1 pyrrolidinone

amidoethylamine (MW=2690) (600 mg, 0.22 mmol, 1.8 mmol amino groups) in 5 mL of MeOH dropwise over 2 – 3 min. This mixture was warmed to RT and stirred for 24 h. The reaction mixture was spotted on a TLC plate and stained with ninhydrin solution to give a negative test. The reaction mixture was added to a Sephadex LH=20 column in MeOH and eluted with a 300 mL void volume followed by 20x20 mL fractions. Fractions 6-12 were found to contain the desired product by spotting each fraction on a TLC plate (silica gel, non-fluorescent) and developing the fractions in an iodine chamber. The volatiles of these fractions were removed using a rotary evaporator to give, after a final evacuation with high vacuum, 770 mg (95% yield) of the title compound (MW=3698) that has the following spectra:

^{13}CNMR(75MHz,D_2O) δ 32.42, 33.73, 34.40, 36.61, 36.24, 36.38, 36.50, 41.95, 48.81, 48.84, 49.51, 50.15, 51.13, 52.90, 174.69, 175.16, 175.42, 175.70.

Example 27: *Linear*-Poly (Amido-pyrrolidone) (PAMPyr) Oligomers

These linear- poly(amidopyrrolidone) **(PAMPyr)** products are analogues to PEGs (Davis *et al., Adv. Drug Delivery Reviews*, **2002,** 54(4), 457-458) and PEOx (Harris *et al.,* US Patent 7,943,141, **2011**) type polymers. As such they exhibit low toxicity, low complement activation features and may be used to reduce protein interactions with drug conjugates while enhancing *in vivo* residency times for these conjugates when used as injectables.

The process to prepare these polymers is as follows.

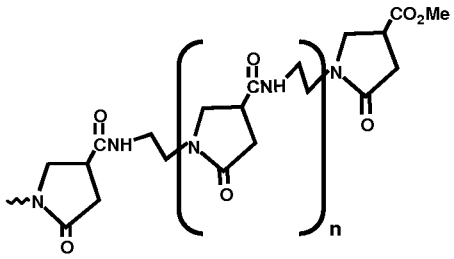
1° Amines / 1°Alkylene diamines + n[DMI+ excess EDA]→ *Linear*-Poly[(Amido-pyrrolidone)]$_n$ (PAMPyr) Oligomers

1. RNH_2 + n (DMI + Excess EDA) → R-NH(*L*-PAMPyr)$_n$ -ester/amine terminated

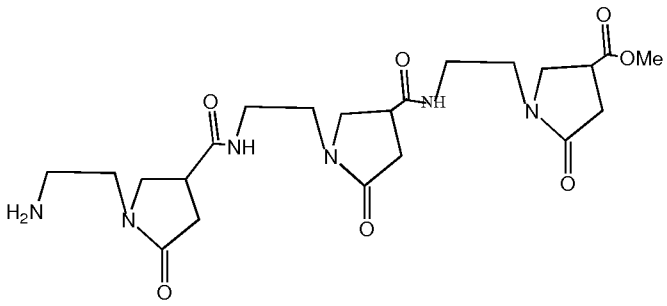
2. BocNHR(X)NH_2 + n (DMI + Excess EDA) → Boc-NH (L-PAMPyr)$_n$ -ester/amine terminated

   (deprotection) → NH_2(L-PAMPyr)$_n$ -ester/amine terminated

3. $NH_2CH_2CH_2$-S-S-$CH_2CH_2NH_2$ + n (DMI + Excess EDA) → [-S-CH_2CH_2N(L-PAMPyr)$_n$]$_2$ → 2 HS CH_2CH_2N(L-PAMPyr)$_n$



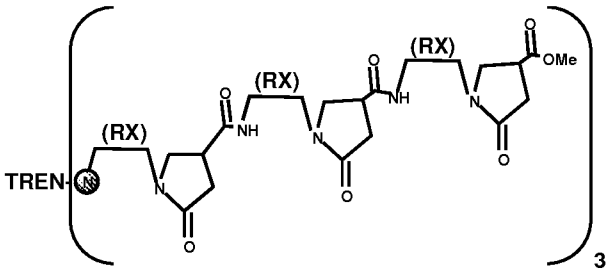
4. (L-PAMPyr)$_n$ -ester/amine terminated



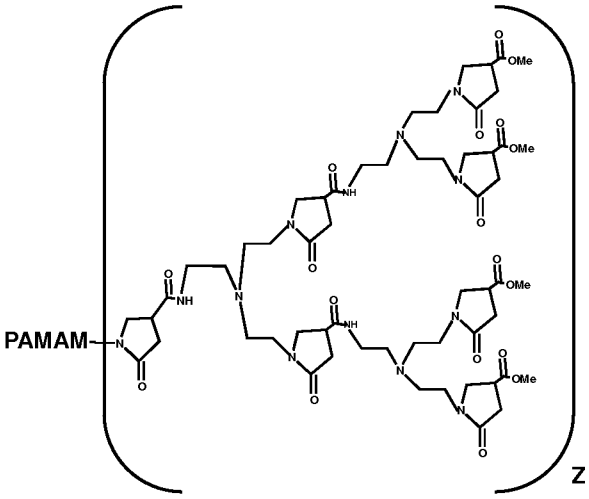
Example 28: Star Branched, *Linear*-Poly (Amido-pyrrolidones) (PAMPyr)

1. N[(CH_2CH_2)NH_2)]$_3$ (TREN) + n (DMI + Excess EDA) →

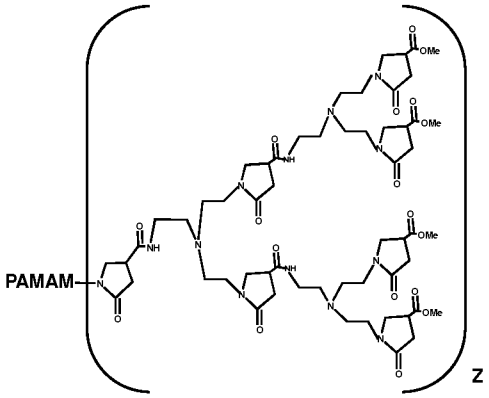
Dendritic, Poly(amido-pyrrolidone) Amines (PAMPyrAM)



2. N[(CH_2CH_2)NH_2)]$_3$ (TREN) + n (DMI + Excess TREN) →



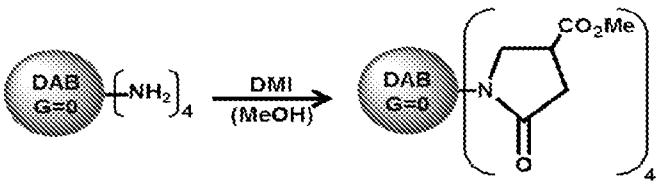
3. PAMAM-$(NH_2)_Z$ + n (DMI + Excess TREN) →



Example 29: Pyrrolidone Terminated PAMAM Dendrimers

Surface functionalized:

Dendrimers (1°amine terminated) + DMI→



Utility Discussion

These new compounds are MW controlled heterocyclic/macrocyclic (*i.e.* pyrrolidone) type polymer compositions that exhibit IF, low toxicity and low complement activation properties that are suitable for *in vivo* biological injection and imaging applications. These polymeric compositions can be prepared in four different architectural forms, namely: (a) linear polymers; (b) cross-linked polymers; (c) branched polymers; and (d) dendritic polymers. This general overview can be seen in Figure 10.

Table 2 below provides the data on the compounds of Formula (I) made by these general procedures. The chemical structures of these compounds are shown in Fig.3 A-D; Comparative example structures are shown in Fig. 4. Additionally, the fluorescence obtained for these compounds is provided.

Table 2

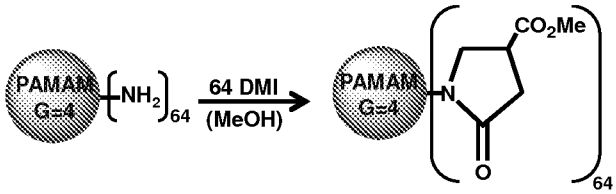
Compound/Example	Excitation Max (nm)	Emission Max (nm)	Relative Response/g
Fluorescein-Na	470	510	1,000,000.0
32	280	340	99,855.2
33	320	380	391.7
40	305	390	66.4
41 peak 1	340	390	62.7
2-Phenyl-2-imidazoline	340	400	50.1
26	325	390	49.8
27	340	400	46.8
39 peak 1	355	415	37.8
3	280	305	36.1
28 peak 1	280	310	33.5
28 peak 2	270	560	31.7
42	335	405	30.7
62	325	410	26.8
43	355	440	22.8
7	340	390	22.4
24	330	410	22.1
30	275	315	18.9
41 peak 2	285	455	18.7
31	300	350	18.7
29	335	400	16.3
G3 DAB Pyrrolidone carboxamidoethylamine	245	410	16.1
11 peak 1	380	480	15.7
G3 DAB Pyrrolidone carboxamidoethylamine pH 2	245	375	15.1
36 peak2	255	305	14.6
44	350	450	14.5
2 peak 1	330	410	14.4
11 peak 2	370	445	14.2

45peak 1	262	285	14.1
46 peak 1	345	405	12.5
G3 DAB Pyrrolidone carboxamidoethylamine	330	410	12.4
2-Pyrrolidone-5-carboxylic acid	245	385	11.8
48	365	435	11.7
47	300	410	11.4
45 peak 2	250 or 270	575	11.2
39 peak 2	245	420	11.1
D	315	380	10.6
G3 DAB Pyrrolidone carboxamidoethylamine pH 2	340	410	10.0
G3 DAB Pyrrolidone carboxamidoethylamine pH 2	200	360	9.3
49 peak 1	260	302	9.0
10	325	410	8.6
9	340	410	8.4
G3 DAB Pyrrolidone carboxamidoethylamine pH 10	200	380	8.3
14	345	430	7.7
37 peak 1	268	302	7.0
Core shell tecto(dendrimer) 1	360	430	6.8
Core shell tecto(dendrimer) 2	360	430	6.6
37 peak 2	260	565	6.5
36 peak 1	340	410	6.4
G3 DAB Pyrrolidone carboxamidoethylamine pH 10	245	380	6.3
50 peak 1	365	440	6.3
51peak 1	305	370	6.3
46 peak 2	260	280	6.2
35	277	301	5.8
2 peak 3	250	415	5.8
51 peak 2	300	350	5.8
G3 DAB Pyrrolidone carboxamidoethylamine pH 10	340	420	5.7
6	260	460	5.4
13 peak 1	335	400	5.0
13 peak 2	295	310	4.7
11 peak 3	265	450	4.4
52	340	410	4.0
(A) PVP MW 40,000	240	385	4.0
20 peak 2	270	440	3.7
18	340	425	3.6
53	340	430	3.4
54	365	440	3.4
55 peak 1	340	410	2.9
2 peak 1	380	460	2.8
38	335	410	2.7
36 peak 2	355	410	2.5
56	335	455	2.5
61	340	420	2.4
36 peak 1	365	445	2.3
16	365	440	2.2
34	360	440	2.1
57 peak 1	340	420	2.1

57 peak 2	285	315	1.9
49 Peak 2	365	450	1.7
56	315	380	1.7
25	350	440	1.6
54 weaker peak	270	435	1.2
5	320	450	1.1
58	365	455	1.0
22	380	450	0.8
59	280	332	0.7
60	340	440	0.7
55 peak 2	305	330	0.7
23	385	460	0.3

When one of these compounds - is bound to a dendrimers and tested for biological purposes in various cell lines, the results are provided in Example I below.

Example I: Synthesis of PAMAM pyrrolidone terminated dendrimer



This dendrimer was tested in the following methods to show utility.

Utility *in vivo*

Cell culture

Chinese hamster fibroblasts (B14) and embryonic mouse hippocampal cells (mHippoE-18) were grown in DMEM medium supplemented with 2 mM glutamine and 10% (v/v) of FBS. Rat liver-derived cells (BRL-3A) were grown in HAM's F12 modified medium, supplemented with 2 mM glutamine and 10% (v/v) FBS. Cells were cultured in T-25 culture flasks in a humidified atmosphere containing 5.0% CO_2 at 37°C and subcultured every 2 or 3 days. Cells were harvested and used in experiments after obtaining 80-90% confluence. The number of viable cells was determined by the trypan blue exclusion assay with the use of Countess Automated Cell Counter (Invitrogen). Cells were seeded either in flat bottom 96-well plates at a density of $1.0x10^4$ cells/well in 100 µL of an appropriate medium or in flat bottom 12-well plates at a density of $2.5x10^5$ cells/well in 1.0 mL of an appropriate medium. After seeding, plates were incubated for 24 h in a humidified atmosphere containing 5.0% CO_2 at 37°C in order to allow cells attaching to the plates.

Uptake and efflux detection

In vitro uptake studies were carried out using autofluorescent G4 PAMAM-pyrrolidone dendrimers (prepared in Example I). Briefly, the dendrimer was added at a concentration of 100 μM to the 24-well plates containing cells at the density of $1.0x10^5$ cells/well. In the uptake study, cells were incubated with the dendrimer for a specific time in a range from 5 min to 48 h in humidified atmosphere containing 5.0% CO_2 at 37°C. In the efflux study, cells were incubated with the dendrimer for 24 h. Then the dendrimer was removed, cells were washed with PBS and incubated further in medium for a defined time in a range from 5 min to 48 h in humidified atmosphere containing 5.0% CO_2 at 37°C. After the appropriate incubation period, cells were washed with PBS, suspended in 500 μL of medium and immediately analyzed with a Becton Dickinson LSR II flow cytometer (BD Biosciences, USA) using a violet laser - 405 nm and Pacific Blue bandpass filter - 450/50nm.

Confocal microscopy

Confocal microscopy images were obtained under 6300x magnification with Zeiss LSM 780 microscope equipped with 405 nm laser diode and InTune excitation laser system (Carl Zeiss Micro Imaging, USA). Cells were grown on 96-well glass-bottom plates and incubated with 100μM G4-PAMAM-pyrrolidone dendrimers (prepared in Example I) for 24 h in 37°C humidified atmosphere containing 5.0% CO_2. After the incubation, cells were imaged directly (unwashed dendrimer fluorescence) or after subsequent plasma membrane/nuclear staining. For the latter, cells were cooled on ice and washed once with cold PBS to inhibit endocytosis. Cell membranes were than stained by 2-min incubation with NeuroDiO carbocyanine dye diluted 200 times in PBS. Due to high lipophilicity of the dye, staining in these conditions may not be uniform. After membrane staining, cell nuclei were stained with RedDot1 nuclear dye diluted 200 times in PBS for 10 min and fixed with 3.6% formaldehyde solution for 15 min in RT. Eventually, fixed and triple stained cells were imaged to visualize intrinsic fluorescence of PAMAM-pyrrolidone dendrimer in blue channel (excitation 405nm, emission 410-470nm), plasma membranes in green channel (excitation 490nm, emission 510-575nm) and nuclei in far-red channel (excitation 595nm, emission 600-740nm).

Results

To analyze cellular uptake of the dendrimer by flow cytometry, cells (B14, BRL-3A, and mHippoE-18) were incubated with the dendrimer at a concentration of 100 μM. It has

been previously shown that the dendrimer at this concentration is not toxic to any tested cell lines (Janaszewska *et al., Nanomedicine NBM,* **2013**, *9*, 461-464). Incubation times varied from 5 min to 48 h. All tested cell lines accumulated PAMAM-pyrrolidone dendrimer rapidly, although its largest amount was observed in mHippoE-18 cells (Fig. 5). After 48 h the intrinsic fluorescence intensity, which is directly proportional to the dendrimer concentration, was almost two times higher for these cells than for B14 cells.

The fluorescence intensity increase that was observed upon incubation of cells with the dendrimer may occur due to two processes: an uptake of the dendrimer within the cells or binding of the dendrimer to the outer layer of cell membranes. To make sure that the dendrimer actually enters the cells and exclude the second possibility, confocal microscopy was used as a visualization technique. Again, this method was based on the intrinsic fluorescence of the dendrimer. The concentration of the dendrimer remained the same and equaled to 100 μM. Confocal images are presented in Fig. 6.

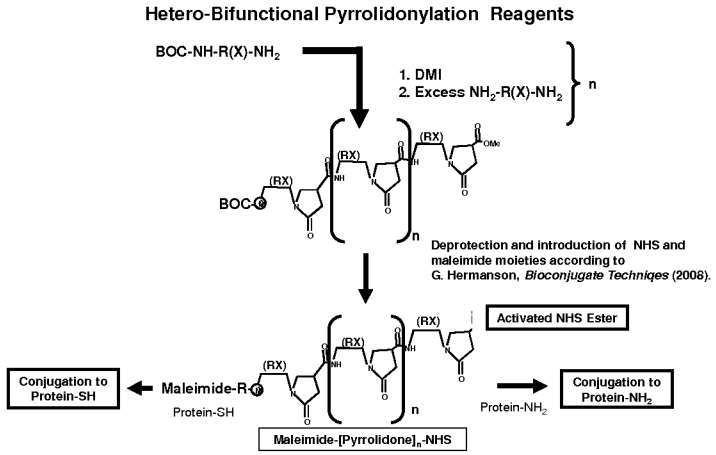
Images showing intrinsic fluorescence of accumulated PAMAM-pyrrolidone dendrimer in three tested cell lines performed after 24 h of treatment without following washout confirm internal localization of the compound (Fig. 6A). Interestingly, some differences in dendrimer localization can be observed between tested cell lines. Although all cells seem to cumulate the dendrimer in cytoplasm, in B-14 and BRL-3A cells nuclear localization can also be observed, whereas in mHippoE-18 cells the fluorescence can be detected in lysosome-like structures, as well as at the cell boundaries (plasma membrane).

In order to further confirm internalization of the dendrimer, all cells were washed once with PBS and stained to visualize plasma membrane and cell nuclei (Fig. 6B). To detect the blue fluorescence of PAMAM-pyrrolidone dendrimer, before formaldehyde fixation, plasma membranes were stained using NeuroDiO carbocyanine dye and nuclei were stained with RedDot1 nuclear dye. As expected, dendrimer fluorescence was localized internally in all tested cell lines. Surprisingly, only BRL-3A cells retained staining pattern observed before dendrimer washout and cell fixation, confirming cytoplasmic and nuclear localization. In B14 cells fluorescence could only be detected in endosome-like structures, lacking nuclear accumulation, similarly to mHippoE-18 cells, where the part of dendrimer fluorescence at the plasma membrane could no longer be observed. It can also be noted that the RedDot1 staining partially colocalizes with the blue fluorescence signal, probably due to non-intended binding of the dye to PAMAM-pyrrolidone dendrimer.

Another important aspect related to dendrimer internalization in cells is knowing the opposite process – their efflux from the cells. Cellular efflux of the studied dendrimer from B14, BRL-3A and mHippoE-18 cells was analyzed analogously as the uptake by flow cytometry based on the dendrimer intrinsic fluorescence. After 24-hour incubation time the dendrimer was removed from the medium, cells were washed and further incubated in a dendrimer-free medium. As it is shown in Figure 7, during the first 24 h the dendrimer was being released from all cells but most slowly from B-14 cells. After 24 h only approx. 15% of dendrimers escaped from the B-14 cells. The fastest leakage was observed for mHippoE-18 cells. In the case of B14 and BRL-3A cells, the amount of uptaken dendrimer was not changing during the first 3 h of the experiment. Nevertheless, after 48 h only a half of initial amount of dendrimer in mHippoE-18 cells, one third in BRL-3A cells and one fifth in B-14 cell was observed.

A modified G4 PAMAM dendrimer with 4-carbomethoxypyrrolidone surface groups referred to as a PAMAM-pyrrolidone dendrimer is characterized by a unique property. It is intrinsically fluorescent in neutral pH without any need of an initial procedure such as oxidation. Moreover, this dendrimer has been found to be very biocompatible and non-toxic, contrary to amino-terminated PAMAM dendrimers (Ciolkowski *et al.*, *Nanomedicine NBM,* **2012**, *8*, 815-817; Janaszewska *et al.*, *Nanomedicine NBM,* **2013**, *9*, 461-464). It even raised a question whether PAMAM-pyrrolidione dendrimer is internalized into cells. Uptake studies combined with confocal microscopy techniques gave a positive answer to this question. Intrinsically fluorescent PAMAM-pyrrolidone dendrimer has been shown to internalize and stain three different cell lines. In the case of BRL-3A cells the dendrimer not only crossed the cell membrane but it also reached the nucleus. To summarize, the PAMAM-pyrrolidone dendrimer possess three distinguishing properties: (1) strong intrinsic fluorescence, (2) low toxicity, (3) cell internalization. Their autofluorescence is strong enough to be visible when dendrimers are in cells.

Due to strong intrinsic blue fluorescence, cellular uptake behavior of PAMAM-pyrrolidone dendrimers could be directly analyzed by confocal microscopy and flow cytometry without additional fluorescence labeling, treatment of dendrimers with chemicals or adjusting pH. This first successful biological experiment opens a broad spectrum of possible PAMAM-pyrrolidone dendrimer applications as gene vectors, and drug delivery platforms that combine two functions: transporting and bioimaging at the same time.



Using routine protocols familiar to those skilled in the art as taught by G.T. Hermanson , *Bioconjugation Techiques,* Second Ed., **(2008)**, these discrete, well defined homo- or hetero-functionalized (see Chapters 4, 5, pp 233-334) PYRROLIDONylation reagents were created to mimic traditional PEGylation reagents. They were prepared from the various new small molecule pyrrolidone derivatives, pyrrolidone containing oligomers, dendrons, dendrimers and polymers described earlier, These PYRROLIDONylation reagents may be conjugated to various proteins, polypeptides, enzymes, antibodies , drugs, polynucleotides, biological substrates, and nanoparticles , etc. as taught by G.T. Hermanason , Chapter 18, 25; in *Bioconjugation Techiques,* Second Ed., **(2008)**, References to these protocols for several specific substrates of interest are as follows: (a) dendrons /dendrimers; Chapter 7, pp 346-394; imaging/chelating agents, Chapter 9,pp400-496; biotin/ avidin, Chapter 11, pp 507-543, Chapter 233, pp 900-921; microparticles/nanoparticles, Chapter 14,15 , pp582-645; hapten-carrier immunogens, Chapter 19, pp745-781; antibodies, Chapter 20, pp787-821; immunotoxins, Chapter 21, pp827-857; liposomes, Chapter 22, pp 858-897; enzymes, Chapter 26. pp 961-968; nucleic acids/oligonucleotides,Chapter 26, pp 970-1002, to mention a few.

This invention now provides a solution to this scientific dilemma by demonstrating that certain interative processes and principles used in the divergent synthesis of dendrimers [*DENDRIMERS, DENDRONS, AND DENDRITIC POLYMERS, Tomalia, D.A., Christensen, J.B. and Boas, U. (**2012**) Cambridge University Press, New York, N.Y.*] may be applied to the synthesis of discrete, well defined poly(pyrrolidone) oligomers and polymers. Figure 10 illustrates the three major architectural components of a dendrimer, namely (a) core, (b) interior and (c) the surface. As such, the divergent construction of a dendrimer

begins with an initiator core around which concentric dendritic layers of branched monomers (i.e., branch cell monomers) are covalently attached in well-defined iterative reaction sequences to produce a sequence of dendritically branched shells referred to as generations (G). This dendritic growth of the interior and the number of surface groups presented as a function of generation is recognized to be mathematically controlled as a function of the core multiplicity (N_c), the branch cell multiplicity, (N_b) and the generation level, (G). As such, the number of surface groups presented at each generation (Z) may be predicted according to the following mathematical expression; $Z=N_cNb^G$. For example, traditional divergent synthesis of poly(amidoamine) (PAMAM) dendrimers begins with an intiator core of know multiplicity(Nc) that may be either an amine or ester. Generally starting with an amine core (i.e., diaminobutane ; (DAB); Nc =4) one adds a stoichiometric amount (i.e., 4X) of methyl acrylate (MA) by Michael addition to produce a PAMAM ester terminated intermediate (Figure 10). This is followed by addition of an excess of ethylene diamine (EDA) which produces a mild, facile amidation of the terminal ester groups to produce the PAMAM amine terminated intermediate (Figure 10). This reaction sequence of (a) Michaels addition followed by (b) amidation constitutes an "iterative reaction sequence " for producing discrete,well-defined dendritic macromolecules referred to as dendrons (Nc=1) or dendrimers (Nc >2).

Earlier we reported the facile reaction of PAMAM dendrimer terminal primary amine moieties to yield N-substituted-4-carbomethoxy pyrrolidones (i.e., (I). Figure 10) [*WO2004/069878, August 19, 2004*], wherein, a precise number of pyrrolidone rings were formed according to the number of primary amines (Z) present as a function of generation. The reaction of primary/secondary amines with the secondary 4-carbomethoxy ester moieties on the pyrrolidone ring were not expected to be and indeed were not as reactive as with the primary ester groups derived from methyl acrylates. That withstanding, we have now found appropriate conditions to perform those reactions in high yield under facile conditions.

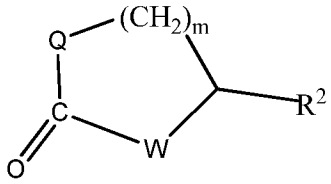
As such, in an effort to control the molecular weights of certain poly(pyrrolidone) oligomers/ polymers below certain threshold limits to assure renal kidney excretion (i.e., <20KDa) for injectable *in vivo* applications or other medical uses, we have invoked the use of certain dendrimer/dendron based "iterative reaction processes /principles". These efforts have now led to the discovery of at least three novel, well-defined poly(pyrrolidone) oligomer/polymer and dendron/ dendrimer compositions (see poly(pyrrolidone composition

categories; II, III and IV (Figure 10) . These compositions exhibit extraordinarily low toxicity, non-complementary activity/ non-interactive, stealthy properties with proteins that are very reminiscent of poly(ethyleneglycols) (PEGs). As such, these compositions are referred to as PYRROLIDONylation reagents and are expected to provide excellent, cost effective alternatives to PEGylation reagents. Furthermore, it was discovered unexpectedly that a non-traditional, "intrinsic fluorescence" property is associated with these pyrrolidone oligomers / polymers as well as many related small molecule pyrrolidone intermediates that have served as monomeric intermediates to these polymeric pyrrolidones.

Although the invention has been described with reference to its preferred embodiments, those of ordinary skill in the art may, upon reading and understanding this disclosure, appreciate changes and modifications which may be made which do not depart from the scope and spirit of the invention as described above or claimed hereafter. Accordingly, this description is to be construed as illustrative only and is for the purpose of teaching those skilled in the art the general manner of carrying out the invention.

WHAT IS CLAIMED IS:

1. Fluorescent cyclic amides, cyclic ureas, cyclic urethanes and cyclic amino amide or amino ureas compounds of the formula



Formula (I)

wherein:

Q is any entity that has a primary amine that reacts to introduce a nitrogen in the ring as shown;

W is N, O, S or $(CH_2)_n$ where n is 0 or 1;

R^2 is -C(O)OH; -C(O)O(C_1-C_4 alkyl); -C(O)-NHR^5 wherein R^5 is C_1-C_4 alkyl or an amido group that can be a moiety on a polymer; and

m is 1-4; and

with the proviso that the compound of Formula (I) fluoresces at least 10x the value of its base compound which is QH_2 not having such cyclic amides, urea, urethanes or cyclic amino amide entities present.

2. The compound of Claim 1 wherein its emissions are in the visible-near infrared region of 400-850 nm.

3. A compound of the formula

$$Q\text{-}(Pyr\text{-}4\text{-}R^2)_x$$

Formula (IV)

wherein:
Q is any entity that has at least one primary amine that reacts to introduce a nitrogen atom into the compound of Formula (IV) and becomes a part of a pyrrolidone moiety;

x is from 1 to the total number of primary nitrogens present in Q;
R^2 is -C(O)OH; -C(O)O(C_1-C_4 alkyl); -C(O)-NHR^5 wherein R^5 is C_1-C_4 alkyl or an amido group that can be a moiety on a polymer; and

with the proviso that the total pyrrolidone moieties have a molecular weight of not greater than 100 KDa and the size of the compound of Formula (IV) is not greater than 15 nm.

4. The compound of Claim 3 that is a MW controlled pyrrolidone moiety containing polymer composition of Formula (IV) that exhibits IF, low toxicity and low complement activation properties that are suitable for *in vivo* biological injection and imaging applications.

5. The compound of Claim 1 or 4 wherein the compound is non-immunogenic to cells.

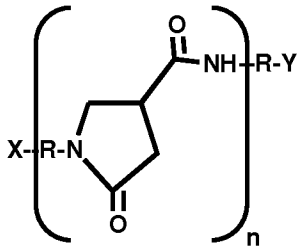
6. The compound of Claim 1 or 3 wherein R^2 is -C(O)O(C_1-C_4 alkyl) or -C(O)OH.

7. The compound of Claim 1 wherein W is $(CH_2)_n$ where n is 1; and m is 1.

8. The compound of Claim 1or 3 wherein Q is an amine terminated dendritic polymer or an amine terminated dendron.

9. The compound of Claim 1 or 3 where Q is (C_1-C_{20} alkyl) amine, (C_1-C_{20} hydroxylalkyl) amine, (C_1-C_{20} alkyl ether) amines, C_6-C_{14} aryl or (C_6-C_{14} aryl C_1-C_4 alkyl) amines.

10. The compound of Claim 1 or 3 where activated PYRROLIDONylation reagents are made having the Formula (III):



Formula (III)

wherein: R is C_1-C_{18} alkyl, C_6-C_{12} aryl, C_6-C_{12} arylene, (C_1-C_{12} alkyl) C_6-C_{12} arylene; and

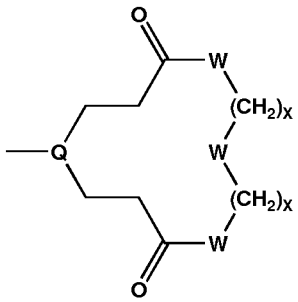
X =Y is independently H, -OH, -NH_2, -SH, –CO_2H, alkyne, azido (*i.e.*, click reagents) or any suitable reactive moiety/ derivative, required for conjugating the PYRROLIDONylation reagents to desired/targeted substrates such as peptides, proteins, antibodies, enzymes, polynucleic acids, drugs, nanoparticles, microparticles, liposomes, micelles, dendrimers, dendrons, dendrimersomes, pharmaceutics, or other biological entities or fragments thereof.

11. Pharmaceutically acceptable salts and esters of $-CO_2H$ are also included in Claims 1, 4 and 10.

12. A method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using a compound of Formula (I) as defined in Claim 1.

13. A method of using the compounds of Claim 4 or 10 for as a substitute for PEG in PEGalaytion.

14. Fluorescent macrocyclic amides having from 4 to 14 components of the formula



Formula (II)

wherein: Q and W are defined as in Formula (I) in Claim 1; and x is 2 or 3.

15. A method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using a compound of Formula (II) as defined in Claim 14.

16. A process for preparing the compounds of Formula (IV) as defined in Claim 3 which comprises reacting Q, as defined in Claim 3, with a reagent of ITA, ITE or DMI, optionally in methanol, in the desired stoichiometry between the various primary amine moieties and the reagents where the resulting compound of Formula (IV) has the total molecular weight of not greater than 100 KDa, preferably not greater than 50 KDa, of the pyrrolidone moieties and the size of the compound of Formula (IV) is not greater than 15 nm, preferably 10 nm.

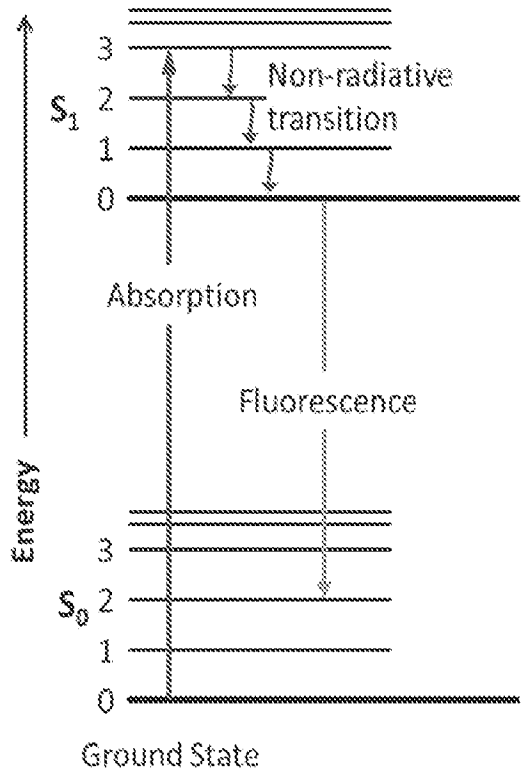

Energy
S_1
3
2
1
0
Non-radiative
transition
Absorption
Fluorescence
S_0
3
2
1
0
Ground State

Figure 1

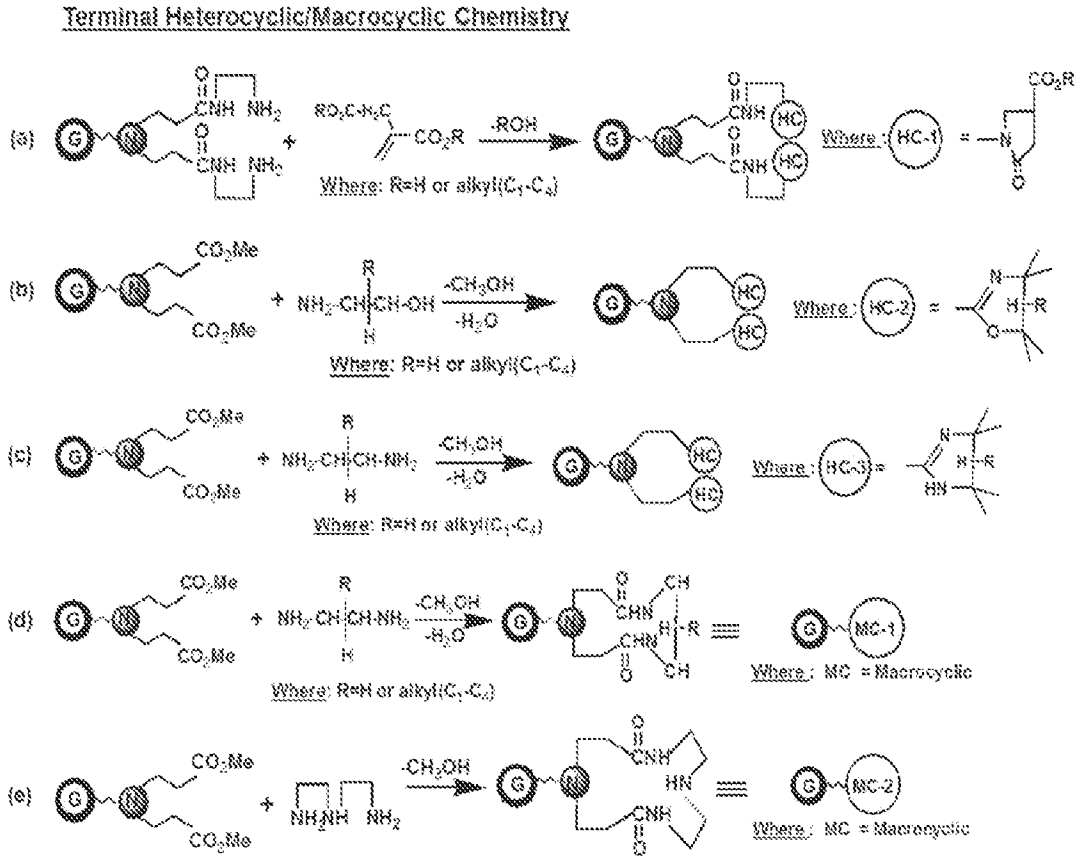

Terminal Heterocyclic/Macrocyclic Chemistry
(a)
-ROH
Where: R=H or alkyl(C1-C4)
Where: HC-1
(b)
-CH3OH
-H2O
Where: R=H or alkyl(C1-C4)
Where: HC-2
(c)
-CH3OH
-H2O
Where: R=H or alkyl(C1-C4)
Where: HC-3
(d)
-CH3OH
-H2O
Where: R=H or alkyl(C1-C4)
Where: MC-1
Where: MC = Macrocyclic
(e)
-CH3OH
Where: MC-2
Where: MC = Macrocyclic

Figure 2

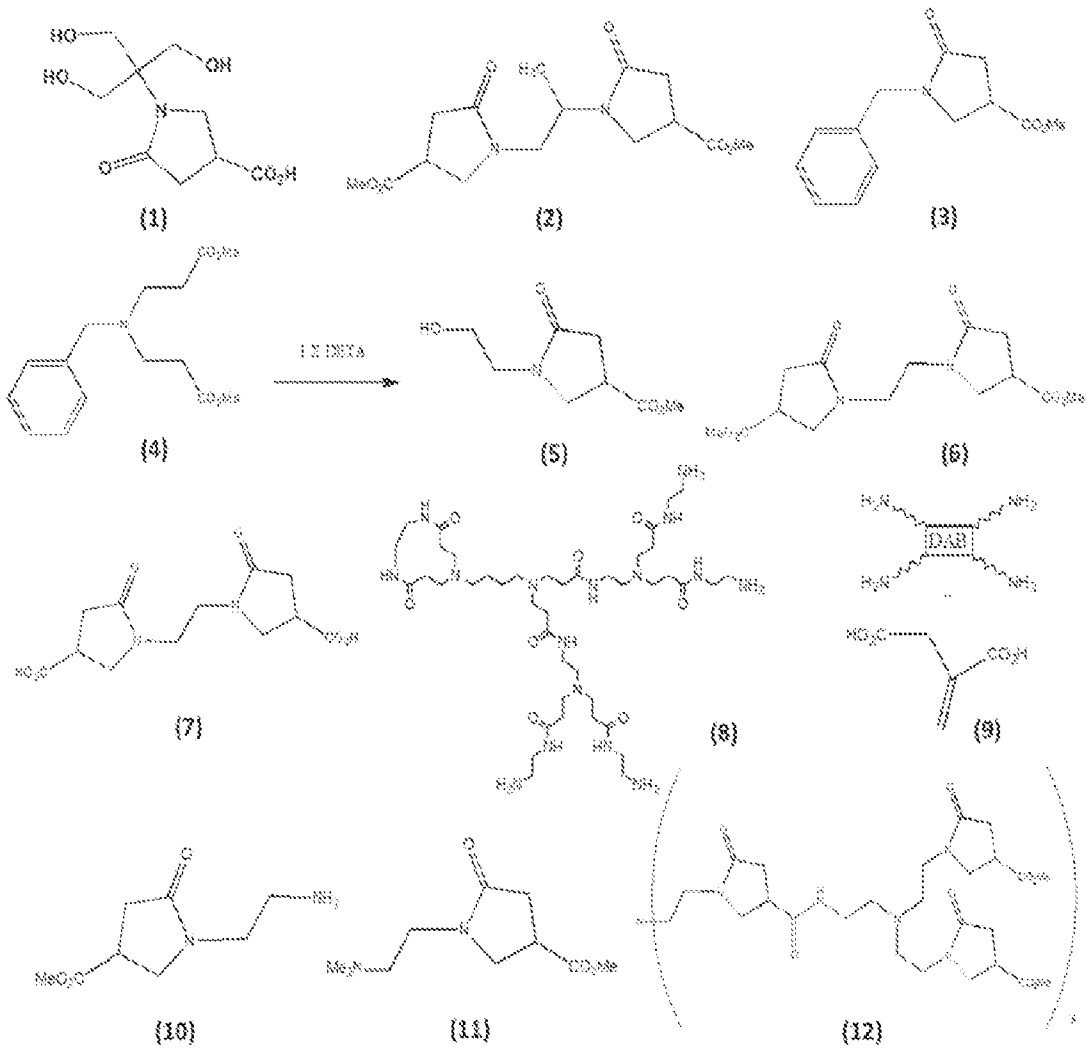


Figure 3A

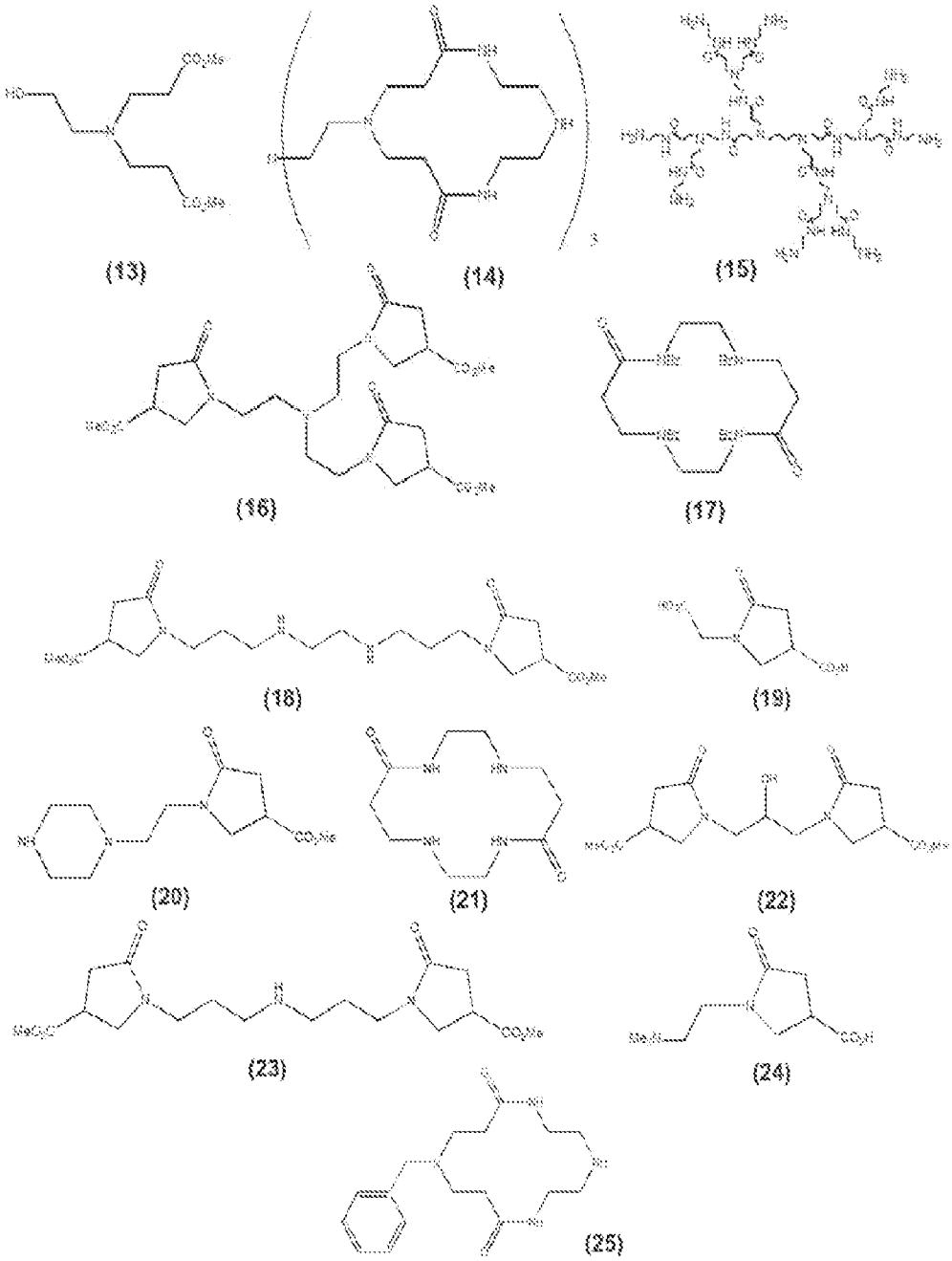


Figure 3 B

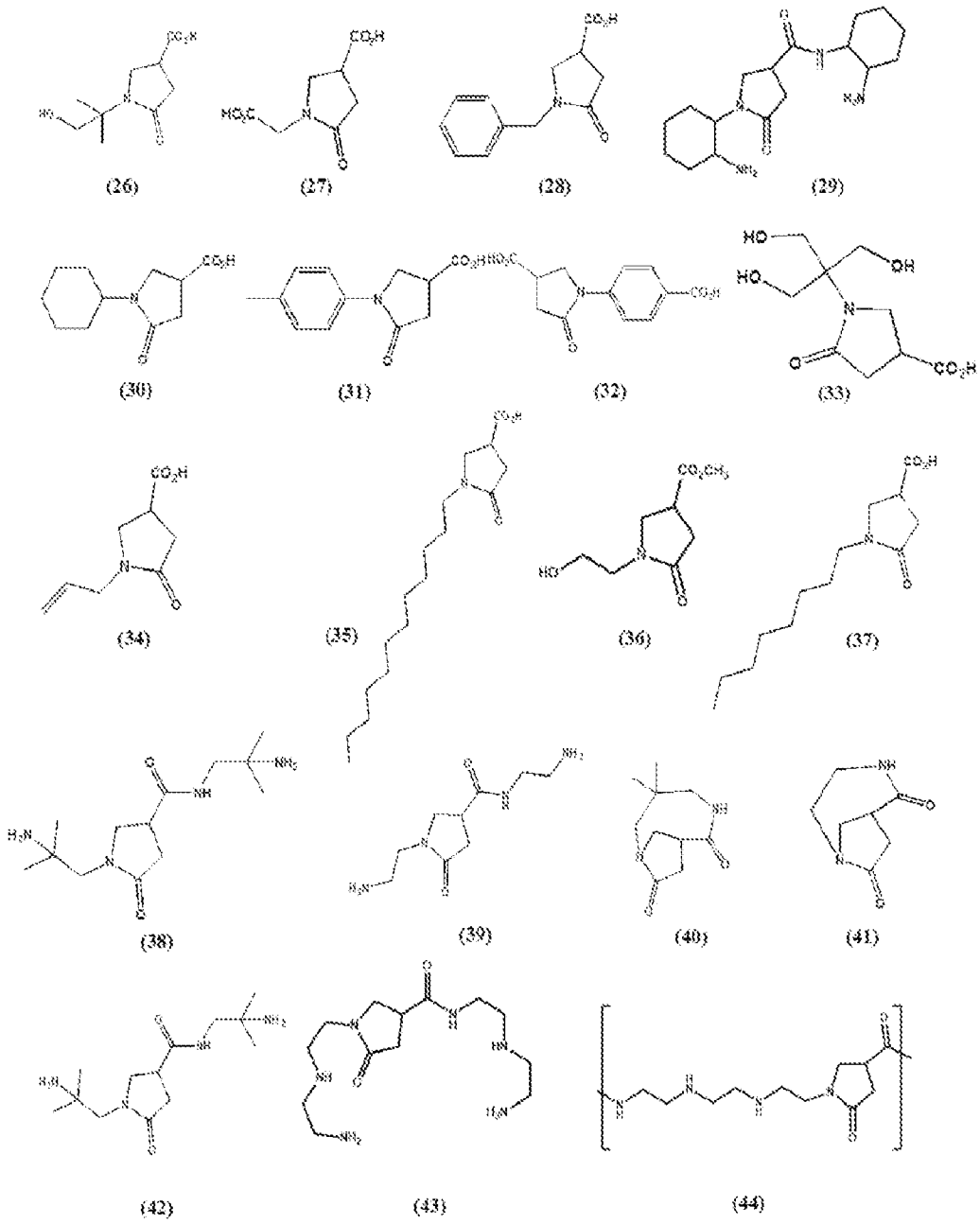


Figure 3 C

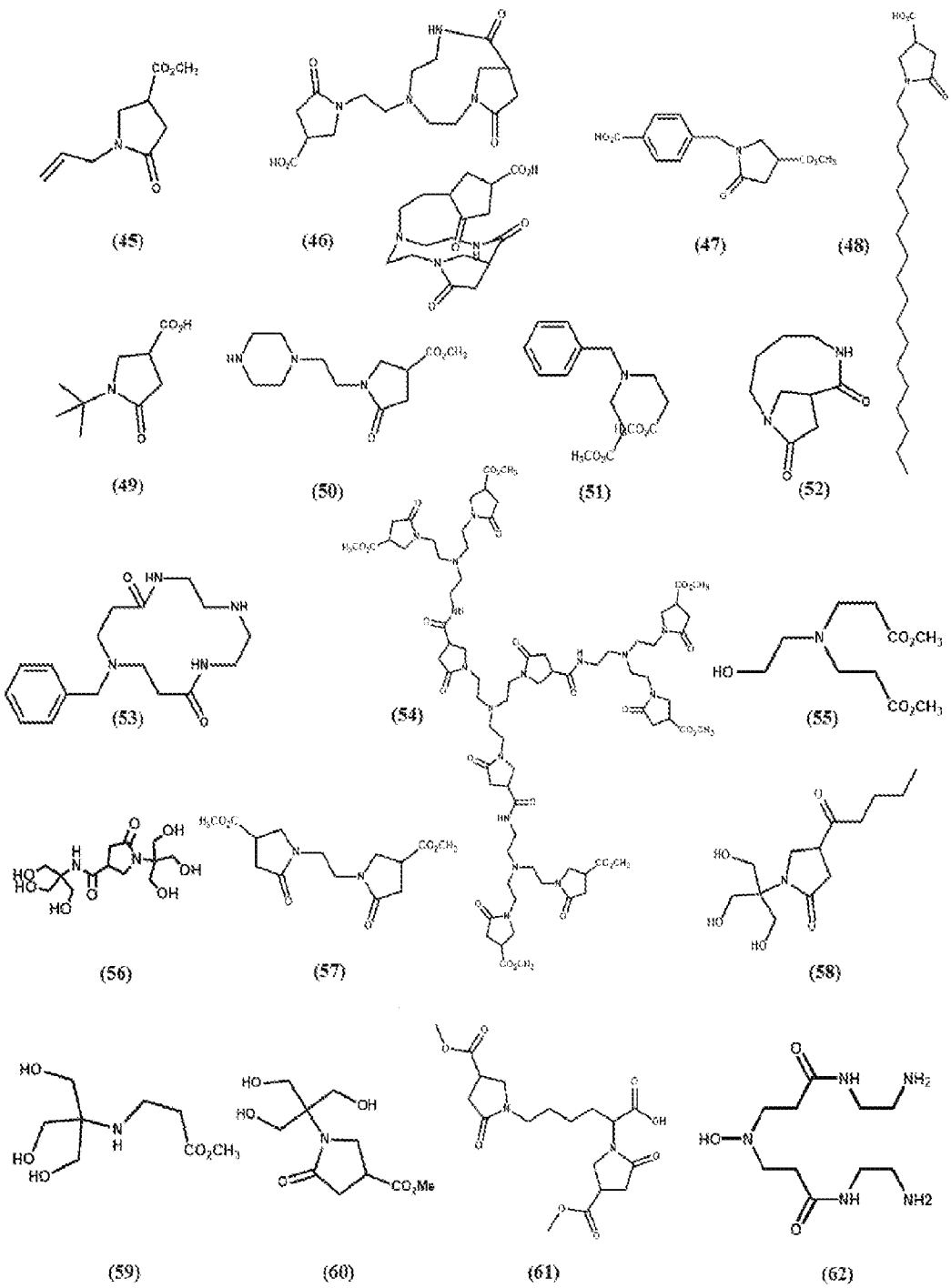


Figure 3D

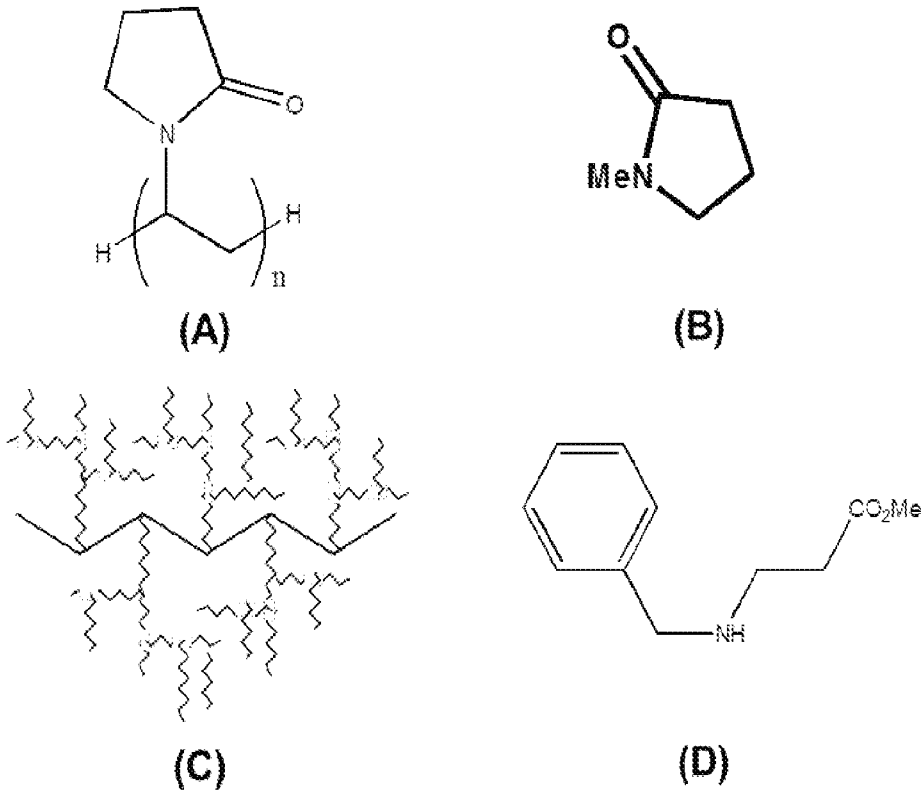
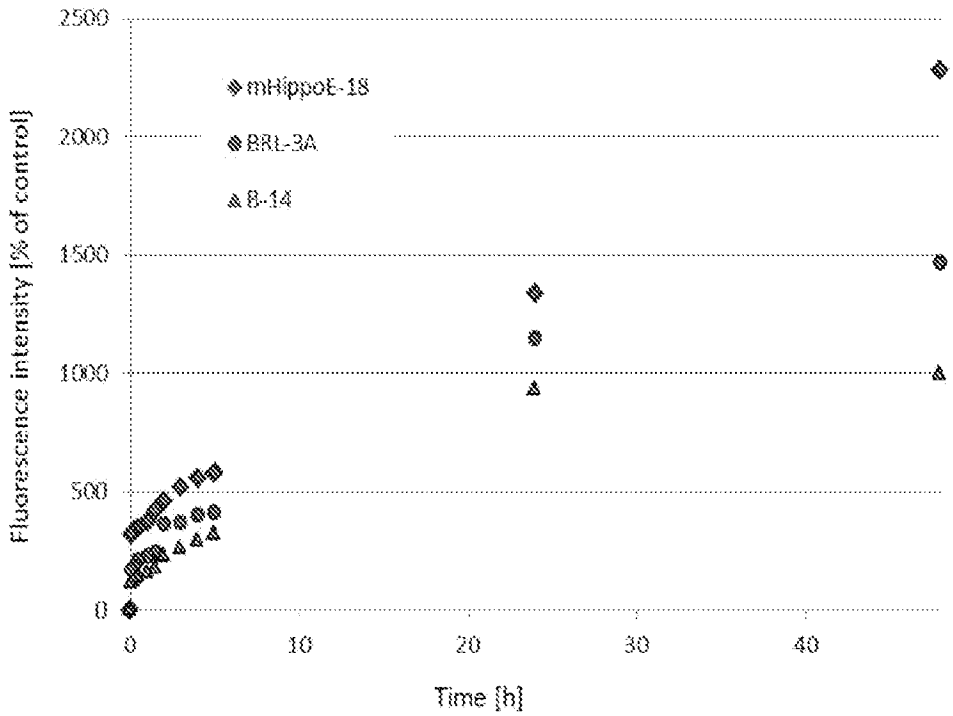


Figure 4



mHippoE-18 (blue rhombus), BRL-3A (red circles) and B-14 (green triangles) cells

Figure 5

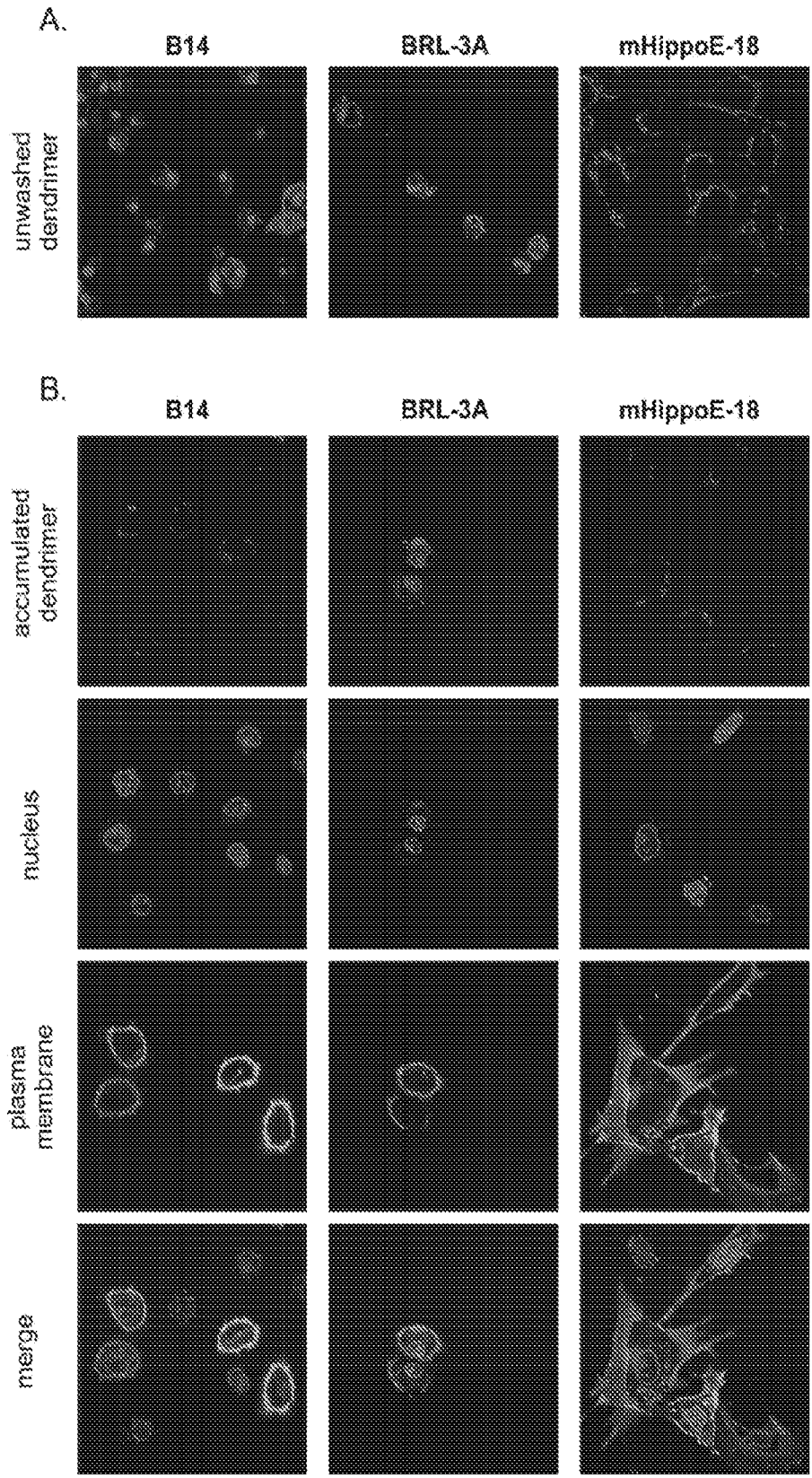
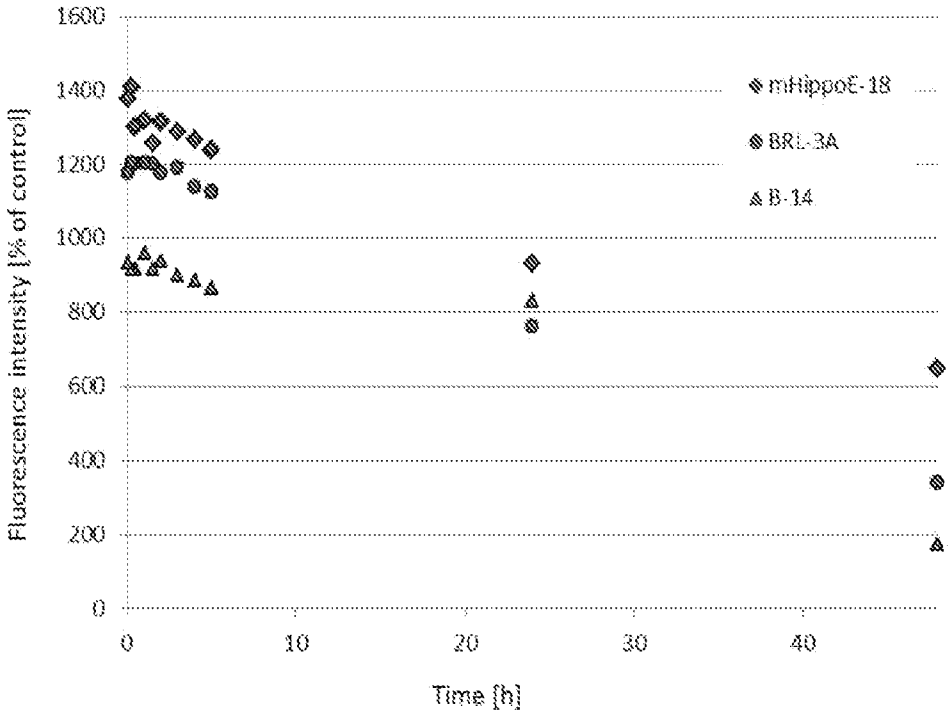

A.
B14
BRL-3A
mHippoE-18
unwashed dendrimer
B.
B14
BRL-3A
mHippoE-18
accumulated dendrimer
nucleus
plasma membrane
merge

Figure 6



mHippoE-18 (blue rhombus), BRL-3A (red circles) and B-14 (green triangles) cells

Figure 7

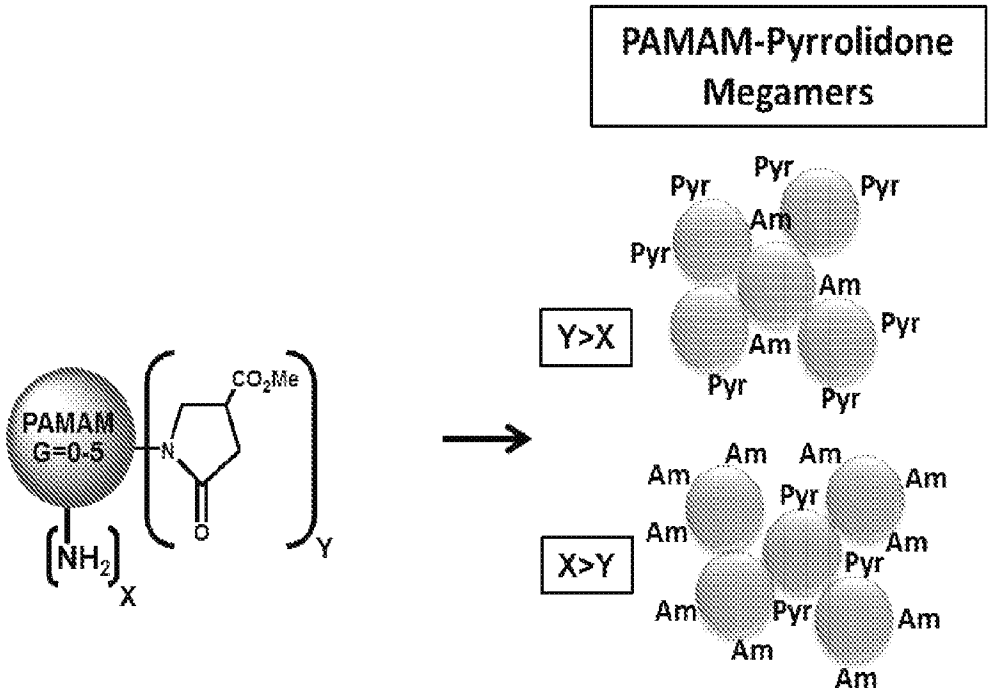
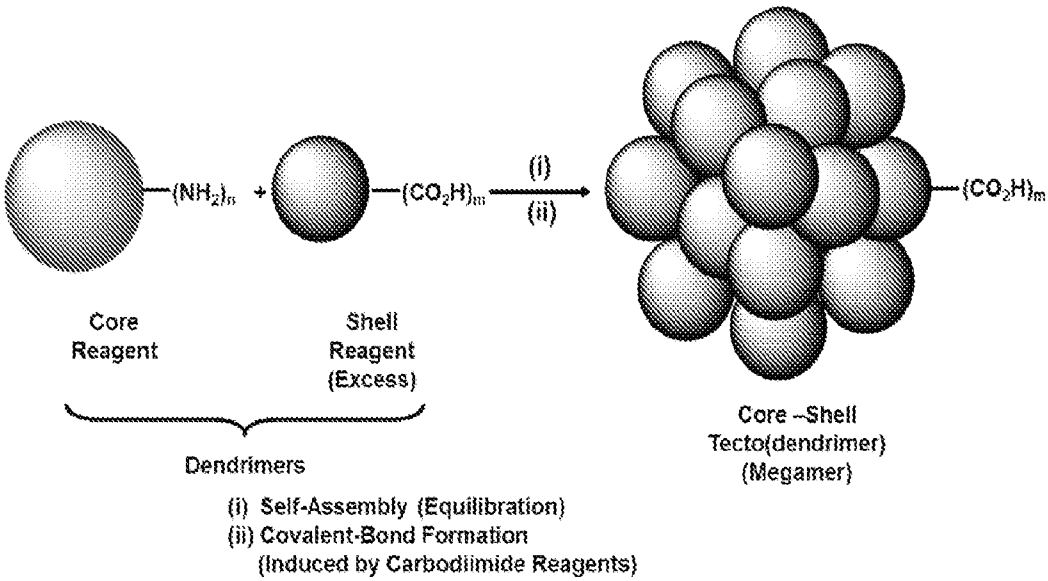
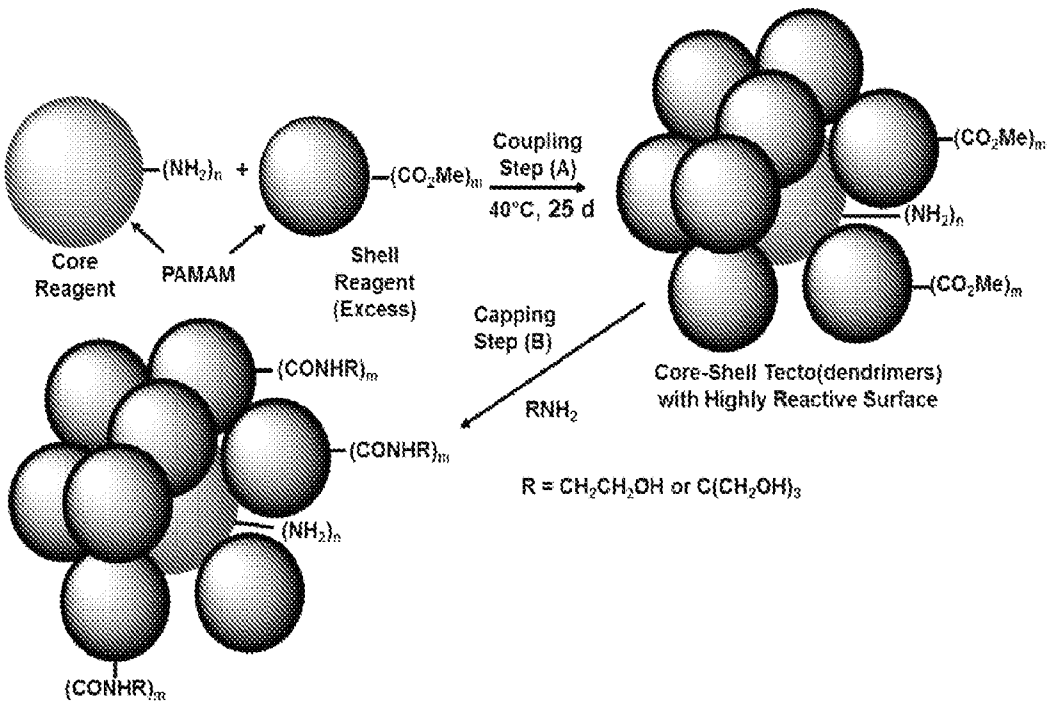

PAMAM-Pyrrolidone Megamers
PAMAM G=0-5
CO_2Me
N
O
NH_2
X
Y
Y>X
X>Y
Pyr
Am

Figure 8


(NH2)n
(CO2H)m
(i)
(ii)
(CO2H)m
Core
Reagent
Shell
Reagent
(Excess)
Dendrimers
Core –Shell
Tecto(dendrimer)
(Megamer)
(i) Self-Assembly (Equilibration)
(ii) Covalent-Bond Formation
(Induced by Carbodiimide Reagents)

(A)


(NH2)n
(CO2Me)m
Coupling
Step (A)
40°C, 25 d
Core
Reagent
PAMAM
Shell
Reagent
(Excess)
(CO2Me)m
(NH2)n
(CO2Me)m
Capping
Step (B)
Core-Shell Tecto(dendrimers)
with Highly Reactive Surface
RNH2
(CONHR)m
(CONHR)m
(NH2)n
(CONHR)m
R = CH2CH2OH or C(CH2OH)3

(B)

Figure 9

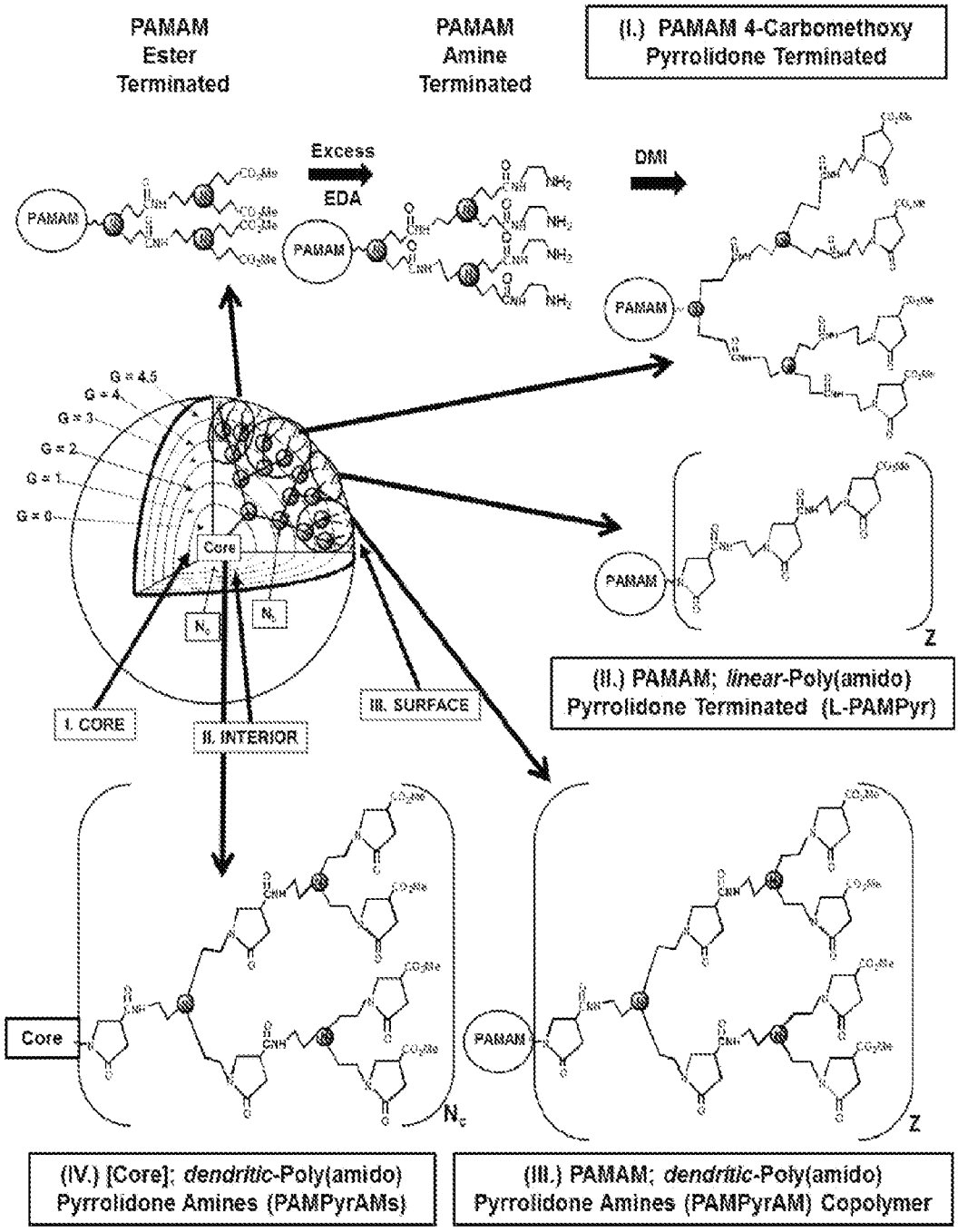

PAMAM
Ester
Terminated
PAMAM
Amine
Terminated
(I.) PAMAM 4-Carbomethoxy
Pyrrolidone Terminated
Excess
EDA
DMI
PAMAM
G = 4.5
G = 4
G = 3
G = 2
G = 1
G = 0
Core
I. CORE
II. INTERIOR
III. SURFACE
(II.) PAMAM; linear-Poly(amido)
Pyrrolidone Terminated (L-PAMPyr)
Core
(IV.) [Core]; dendritic-Poly(amido)
Pyrrolidone Amines (PAMPyrAMs)
(III.) PAMAM; dendritic-Poly(amido)
Pyrrolidone Amines (PAMPyrAM) Copolymer

Figure 10

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/50062

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07D 207/28; C07D 207/277 (2015.01)
CPC - C07D 207/28; C07D 207/277
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C07D 207/28; C07D 207/277 (2015.01)
CPC: C07D 207/28; C07D 207/277

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 548/534; 548/531; 548/537

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PatBase, Google Scholar, PubWEST
pyrrolidone, primary amine, itaconic acid, dimethyl itaconate, polymer, protein delivery, fluorescence

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2006/0160988 A1 (TOMALIA et al.) 20 July 2006 (20.07.2006) Fig 4; Fig 5	1-2, 5-10, 12
Y	CIOLKOWSKI et al. 'Surface modification of PAMAM dendrimer improves its biocompatibility', Nanomedicine: Nanotechnology, Biology and Medicine, Volume 8, Issue 6, August 2012, Pages 815-817. pg 816, Fig 1; Fig 2C; pg 816, col 2, para 2 to pg 817, col 1, para 1; pg 817, col 2, para 2	1-2, 5-10, 12
Y	US 2013/0217892 A1 (WACKER et al.) 22 August 2013 (22.08.2013) para [0008]-[0017], [0060], Example 4	9, 10
A	US 2007/0298006 A1 (TOMALIA et al.) 27 December 2007 (27.12.2007) Entire Document	1-2, 5-10, 12
A	KIM et al. 'Dendrimer Probes for Enhanced Photostability and Localization in Fluorescence Imaging', Biophysical Journal, April 2013, Vol.104, pp 1566-1575. Fig 1; Fig 2	1-2, 5-10, 12

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 January 2016 (15.01.2016)	29 JAN 2016
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/50062

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 11 and 13
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: Claims 1-2, (5-6, 8-10) (in part), 7 and 12 directed to a fluorescent compound of Formula (I), that fluoresces at least 10 times the value of its base compound and to a method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using a compound of Formula (I).

Group II: Claims 3-4, (5-6, 8-10) (in part) and 16 directed to a compound of Formula (IV) having a molecular weight not greater than 100 KDa and to a process of producing the same.

Group III: Claims 14-15 directed to a fluorescent macrocyclic amide compound of Formula (II), and to a method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using a compound of Formula (II). --Please see extra sheet--

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, (5-6, 8-10) (in part), 7 and 12

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 15/50062

Continuation of Box.No.III:

The groups of inventions listed above do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Special Technical Features:

Group I includes the technical feature of a pyrrolidone compound that fluoresces at least 10 times the value of its base compound, not required by Groups II and III.

Group II includes the technical feature of a pyrrolidone compound having a molecular weight not greater than 100 KDa, not required by Groups I and III.

Group III includes the technical feature of a macrocyclic compound having the structure of Formula (II), not required by Groups I and II.

Common technical features:

Groups I-III share the technical feature of a cyclic derivative of a primary amine.

Groups I and II further share the technical feature of a 4-carboxy-pyrrolidone derivative of a primary amine, optionally further derivatized as an ester or amide of the carboxyl.

These shared technical features, however, do not provide a contribution over the prior art, as being anticipated by US 2006/0160988 A1 to Tomalia et al. (hereinafter 'Tomalia') which discloses a 2-pyrrolidone derivative of a primary amine, having the structure of Formula (I), wherein Q is the primary amine; m is 1; W is (CH2)n, wherein n is 1 and R2 is -C(O)OC1-alkyl (Figure 4, scheme).
As said compound was known in the art at the time of the invention, these cannot be considered special technical features, that would otherwise unify the inventions of Groups I-III.

Groups I and III further share the technical feature of a fluorescent cyclic amide compound and of a method of tracing moieties and fluids in various systems, tests, plants or animals and humans by fluorescence, and monitoring or detecting the location of the moiety by fluorescence using said cyclic amide compound.

These shared technical features, however, do not provide a contribution over the prior art as being anticipated by the article entitled, 'Targeting Gelatinases with a Near-Infrared Fluorescent Cyclic His-Try-Gly-Phe Peptide', published in Mol Imaging Biol. 2009 ; 11(6): 424-433, by Wang et al. (hereinafter 'Wang') which discloses a fluorescent cyclic amide compound useful in in vitro or in vivo imaging applications for tracing moieties in animals (abstract; scheme 1; Fig 3; Fig 5). As said compound and use were known in the art at the time of the invention, these cannot be considered special technical features, that would otherwise unify the inventions of Groups I and III.

The inventions of Groups I-III, therefore, lack unity under PCT Rule 13.

Note reg item 4: Claims 11 and 13 are unsearchable because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a). These claims are therefore, not included in the above analysis.