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(54) **BIODEGRADABLE POLY(β-AMINO ESTERS) AND USES THEREOF**

(75) Inventors: **Daniel Griffith Anderson**, Sudbury, MA (US); **David M. Lynn**, Middleton, WI (US); **Akin Akinc**, Needham, MA (US); **Robert S. Langer**, Newton, MA (US)

(73) Assignee: **Massachusetts Institute of Technology**, Cambridge, MA (US)

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See application file for complete search history.

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*Primary Examiner* — Blessing Fubara
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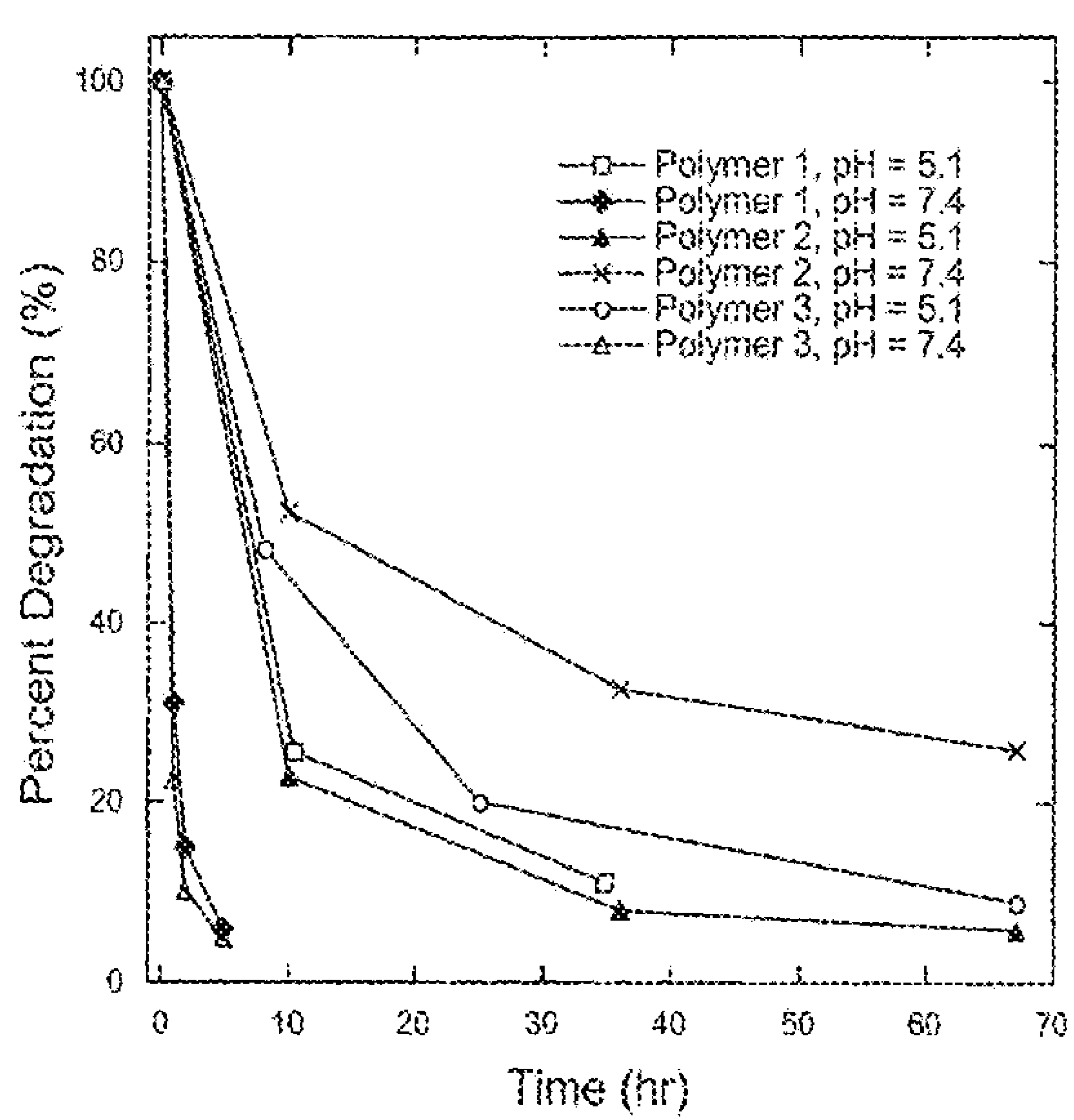
(57) **ABSTRACT**

Poly(β-amino esters) prepared from the conjugate addition of bis(secondary amines) or primary amines to a bis(acrylate ester) are described. Methods of preparing these polymers from commercially available starting materials are also provided. These tertiary amine-containing polymers are preferably biodegradable and biocompatible and may be used in a variety of drug delivery systems. Given the poly(amine) nature of these polymers, they are particularly suited for the delivery of polynucleotides. Nanoparticles containing polymer/polynucleotide complexes have been prepared. The inventive polymers may also be used to encapsulate other agents to be delivered. They are particularly useful in delivering labile agents given their ability to buffer the pH of their surroundings. A system for preparing and screening polymers in parallel using semi-automated robotic fluid delivery systems is also provided.

**67 Claims, 27 Drawing Sheets**



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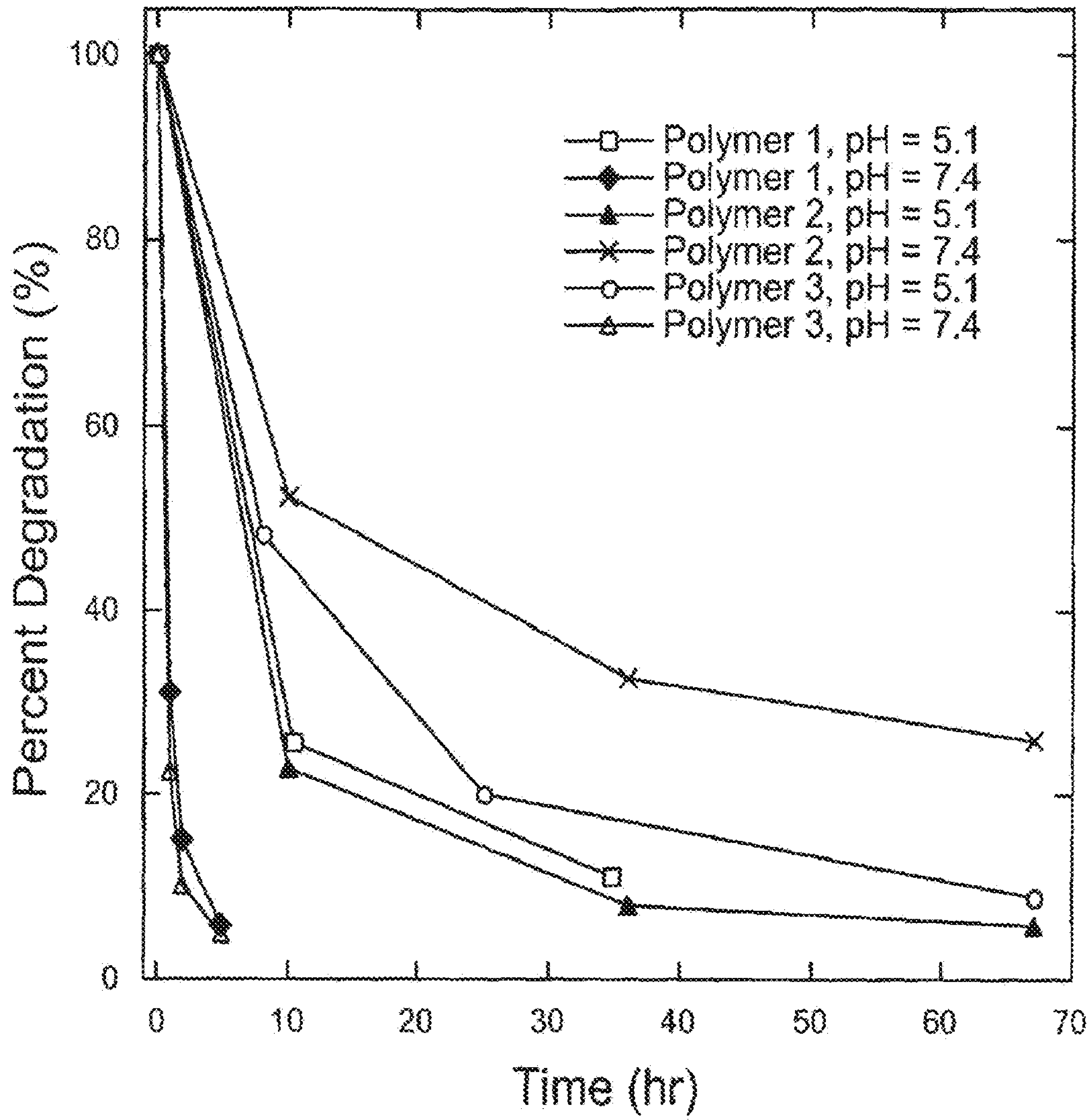

Polymer 1, pH = 5.1
Polymer 1, pH = 7.4
Polymer 2, pH = 5.1
Polymer 2, pH = 7.4
Polymer 3, pH = 5.1
Polymer 3, pH = 7.4
Percent Degradation (%)
Time (hr)
0
20
40
60
80
100
0
10
20
30
40
50
60
70

Figure 1

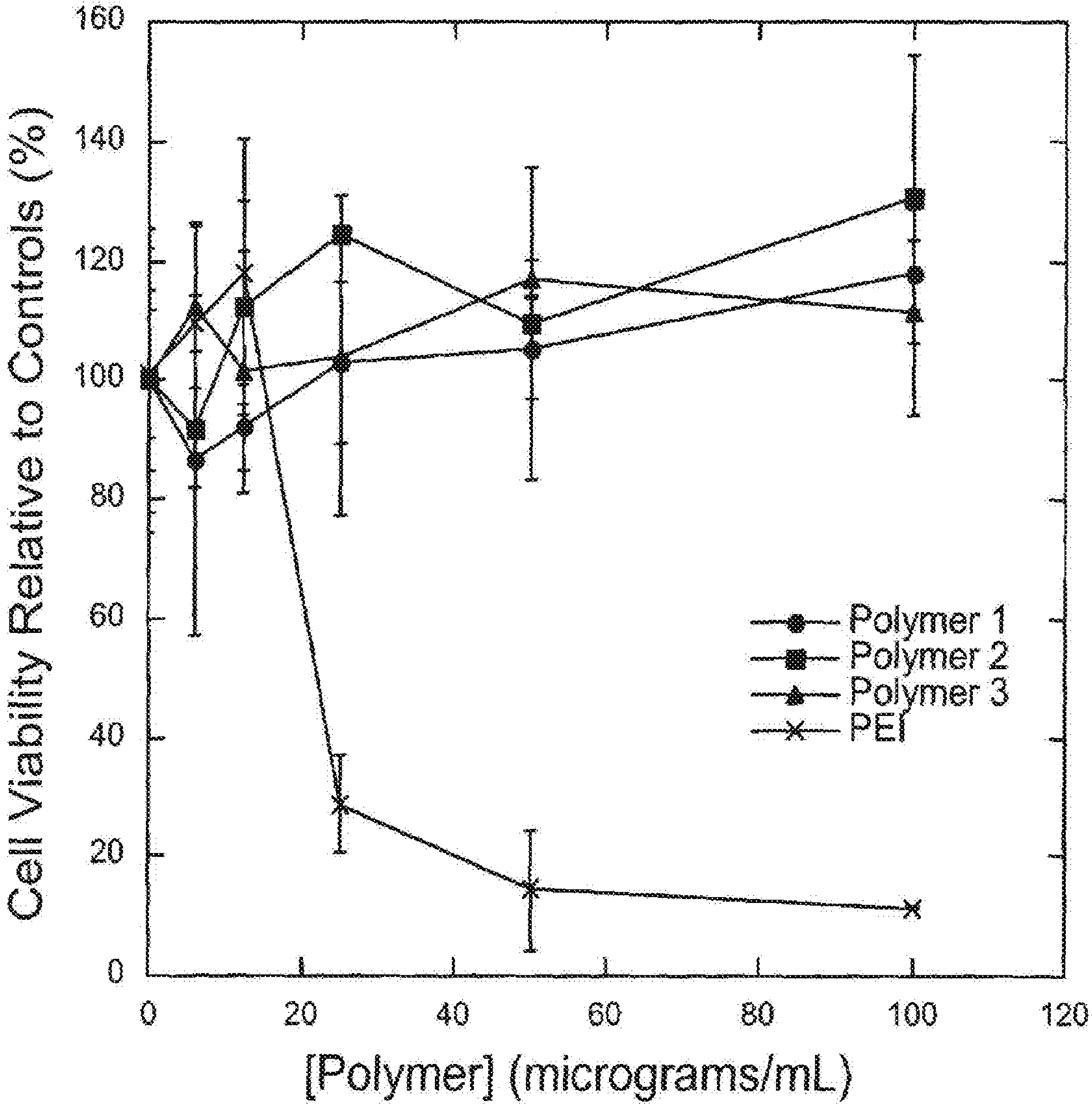

Cell Viability Relative to Controls (%)
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
[Polymer] (micrograms/mL)
Polymer 1
Polymer 2
Polymer 3
PEI

Figure 2

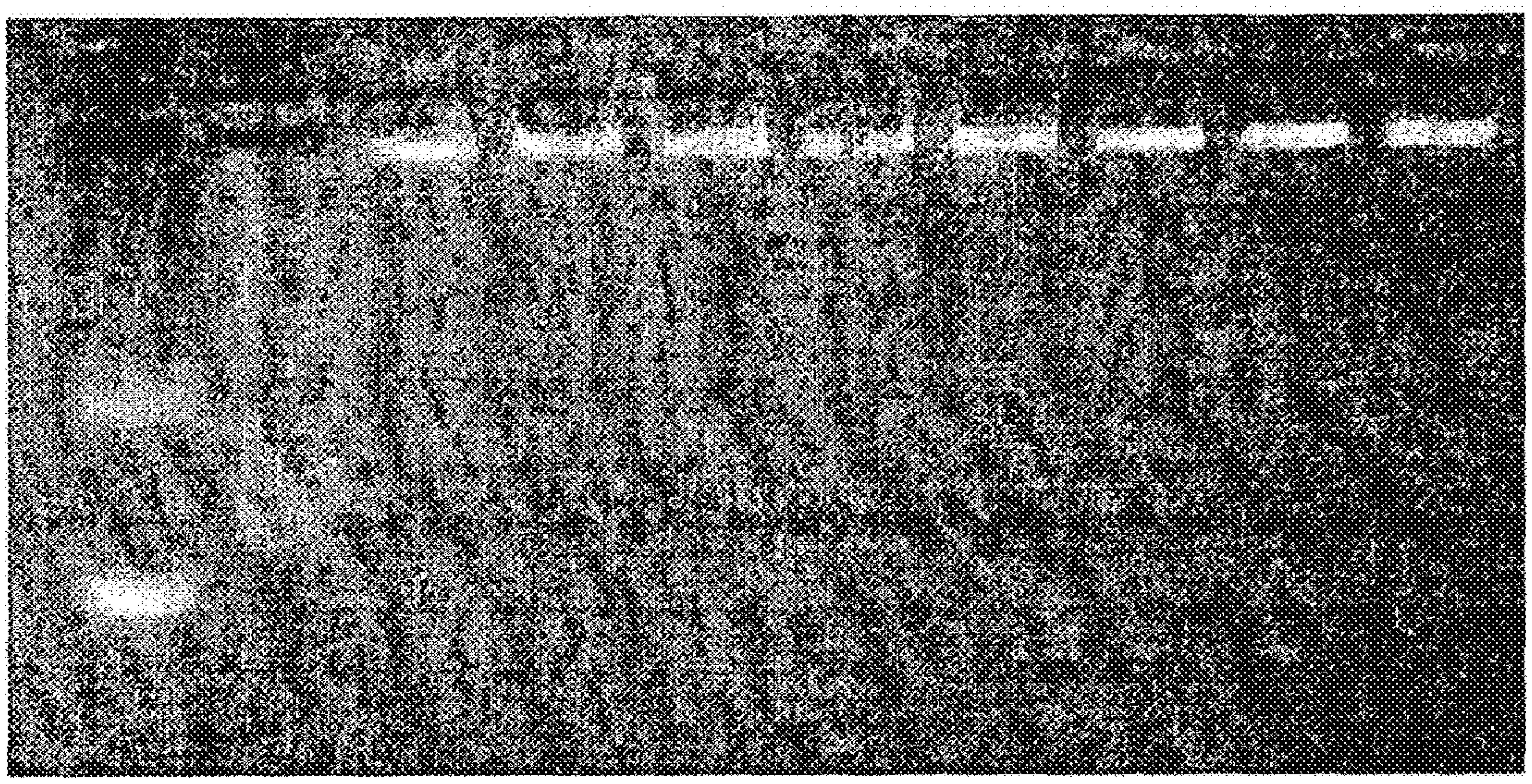
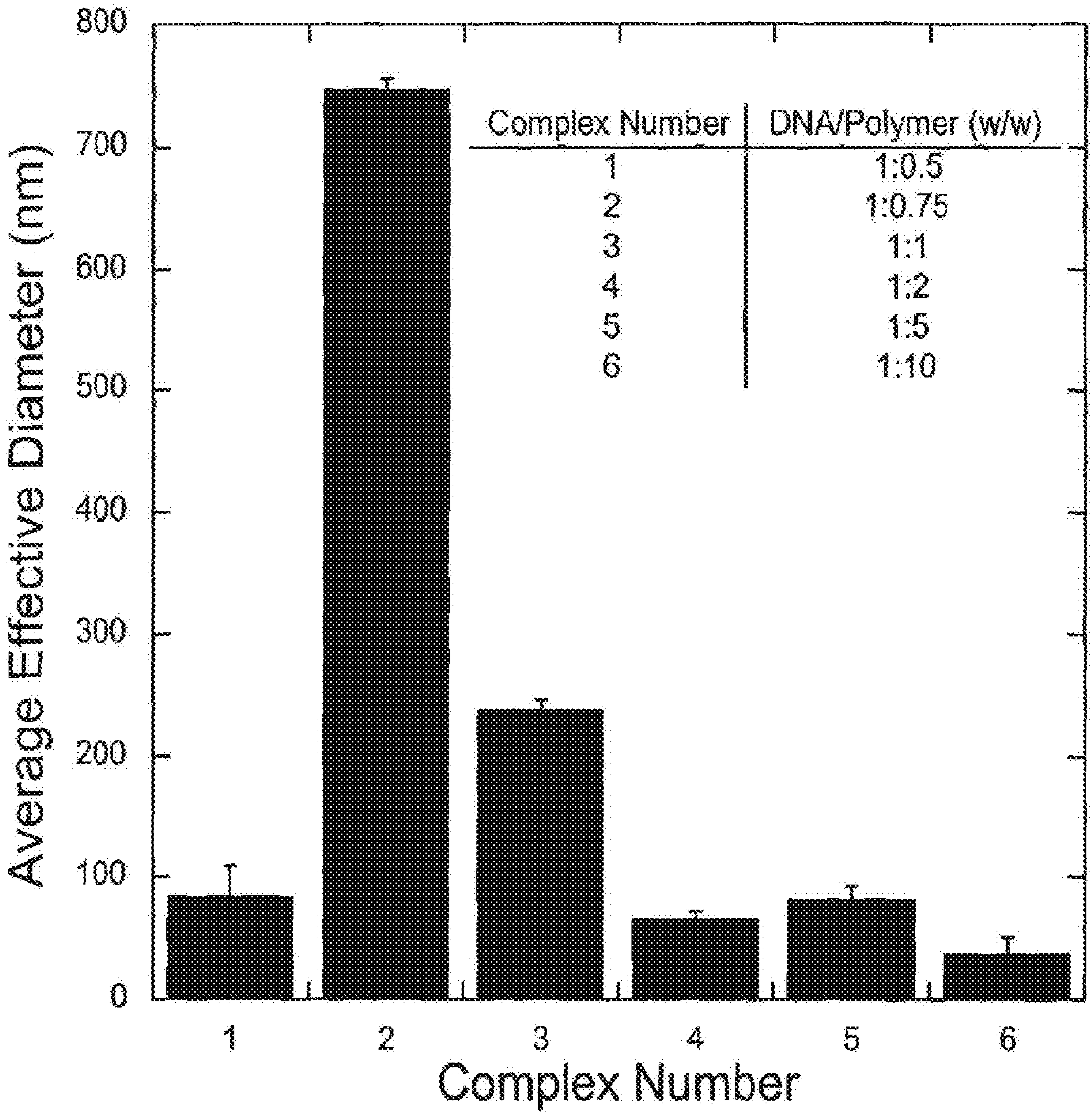
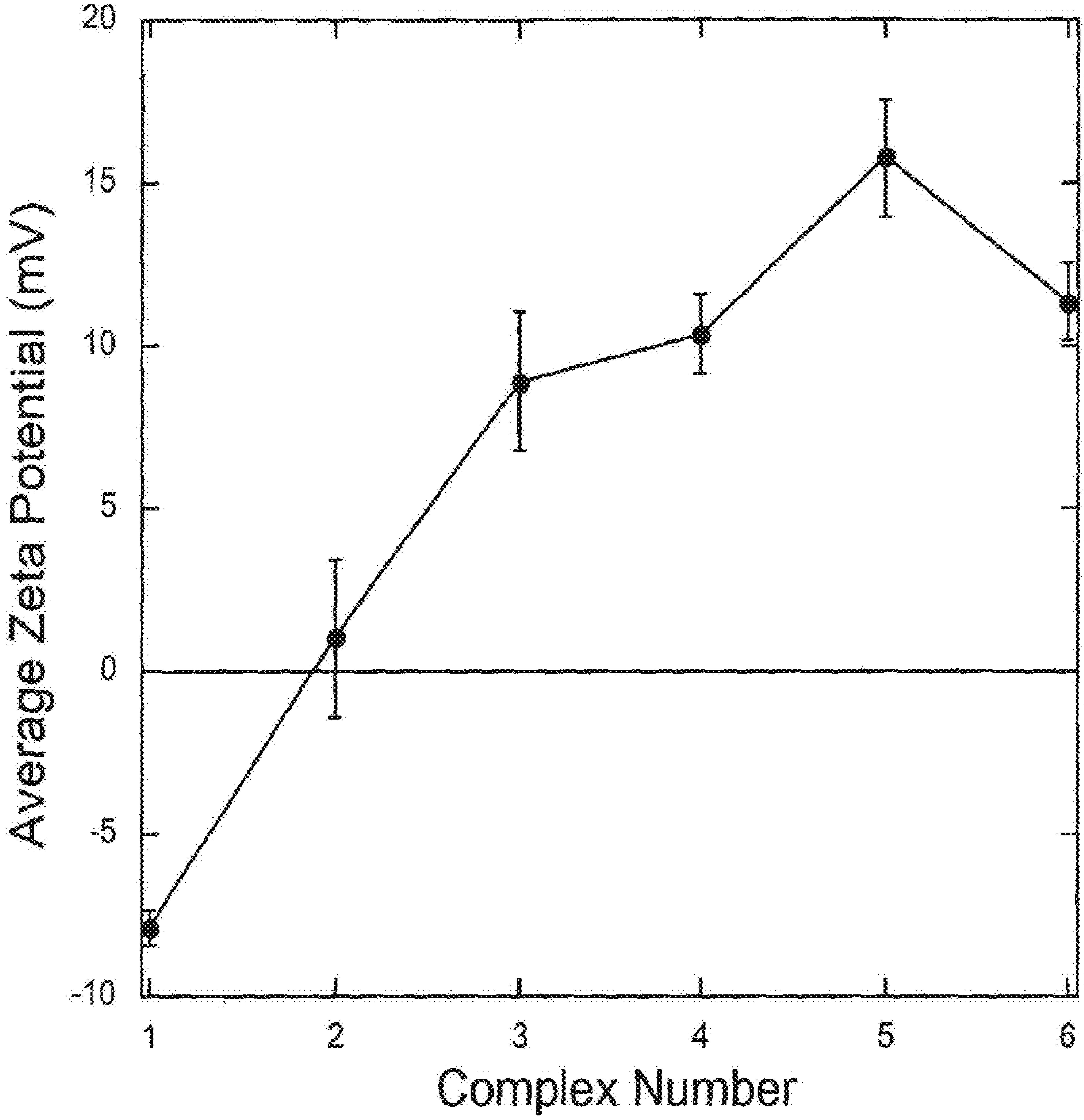


Figure 3



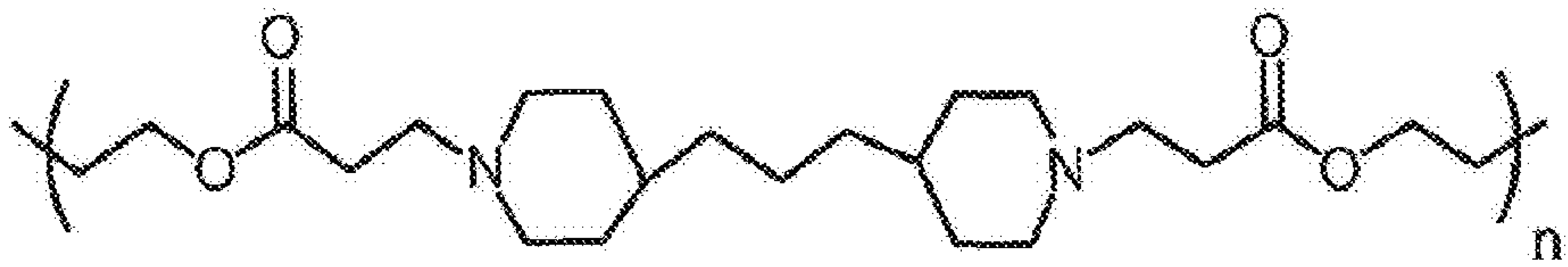
800
700
600
500
400
300
200
100
0
Average Effective Diameter (nm)
Complex Number
DNA/Polymer (w/w)
1 1:0.5
2 1:0.75
3 1:1
4 1:2
5 1:5
6 1:10
1
2
3
4
5
6
Complex Number


Figure 4



Average Zeta Potential (mV)
20
15
10
5
0
-5
-10
1
2
3
4
5
6
Complex Number


Figure 5



poly-1

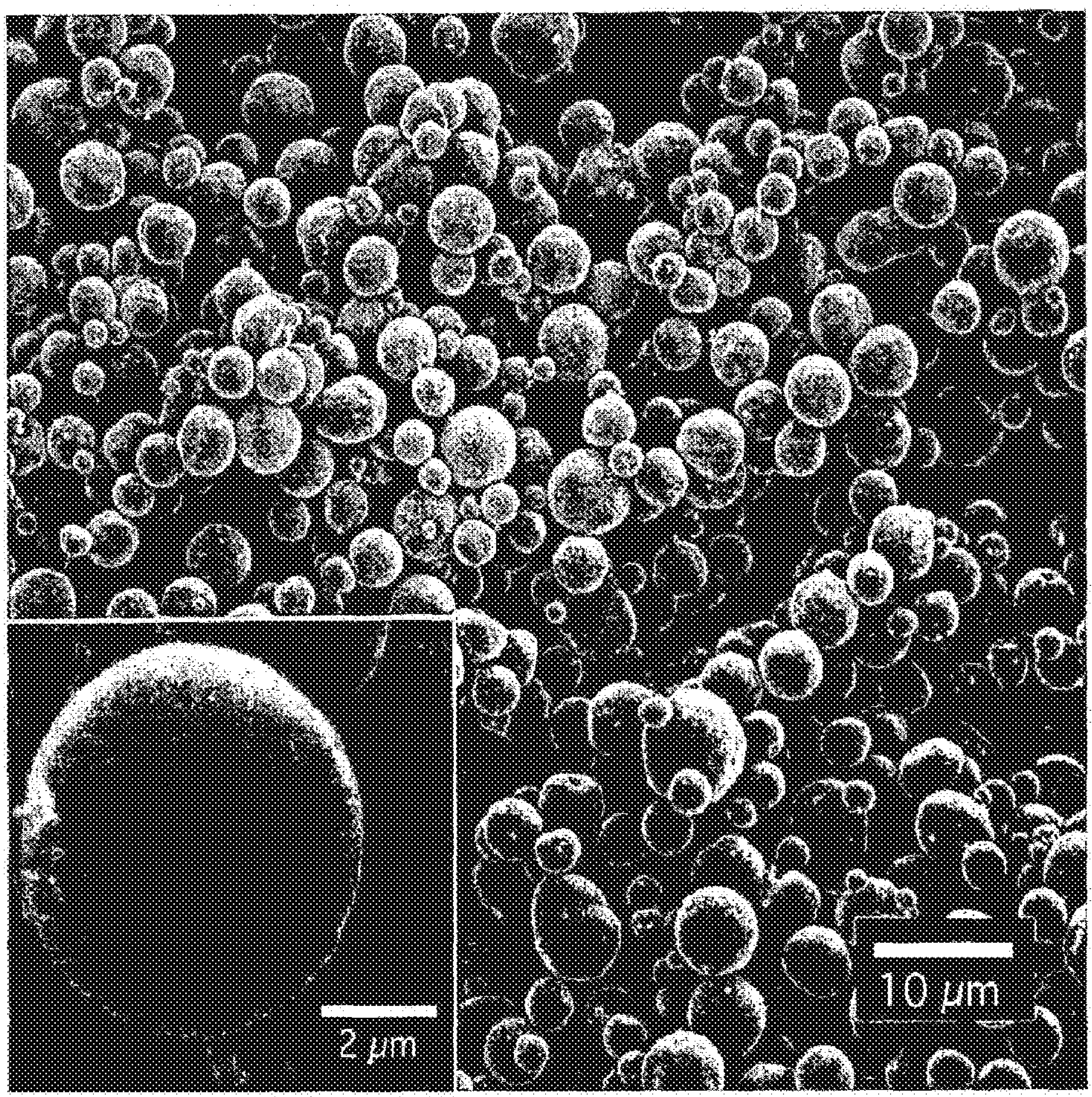



Figure 6

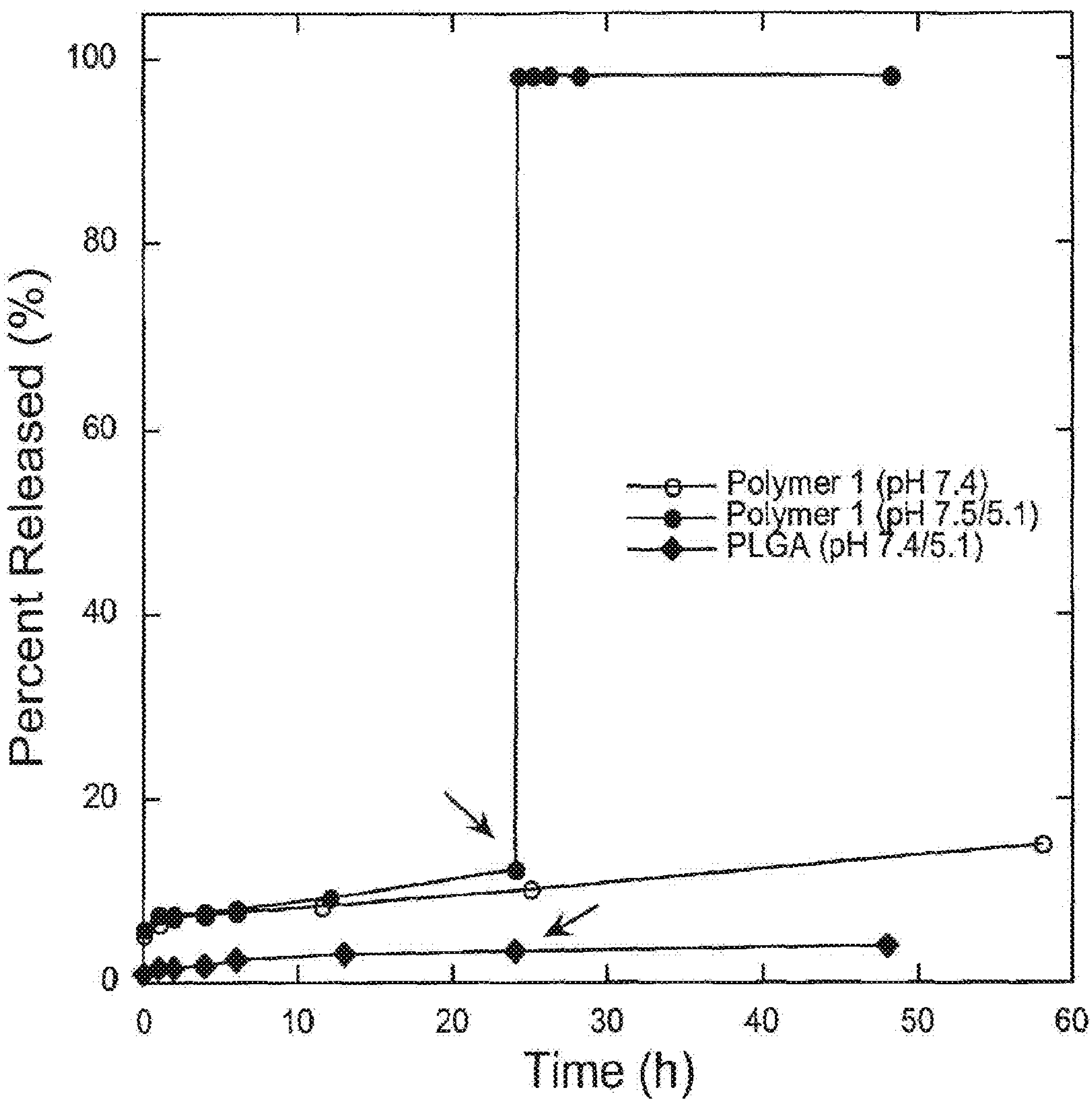

Percent Released (%)
Time (h)
Polymer 1 (pH 7.4)
Polymer 1 (pH 7.5/5.1)
PLGA (pH 7.4/5.1)
0
20
40
60
80
100
0
10
20
30
40
50
60

Figure 7

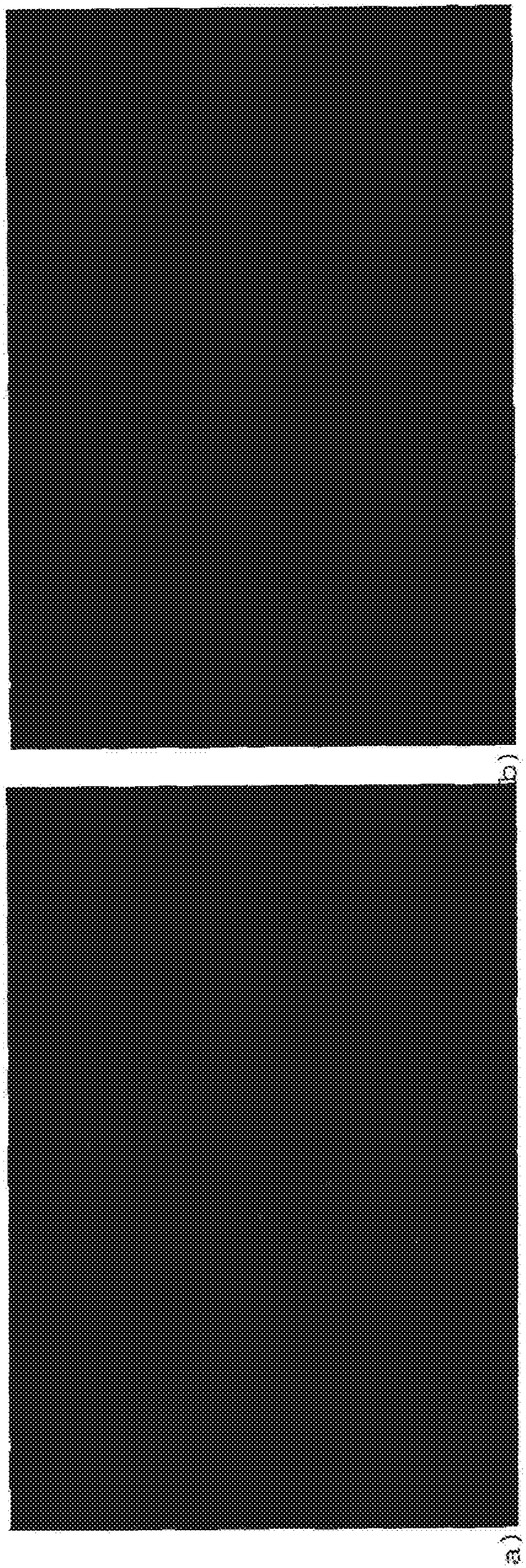



Figure 8

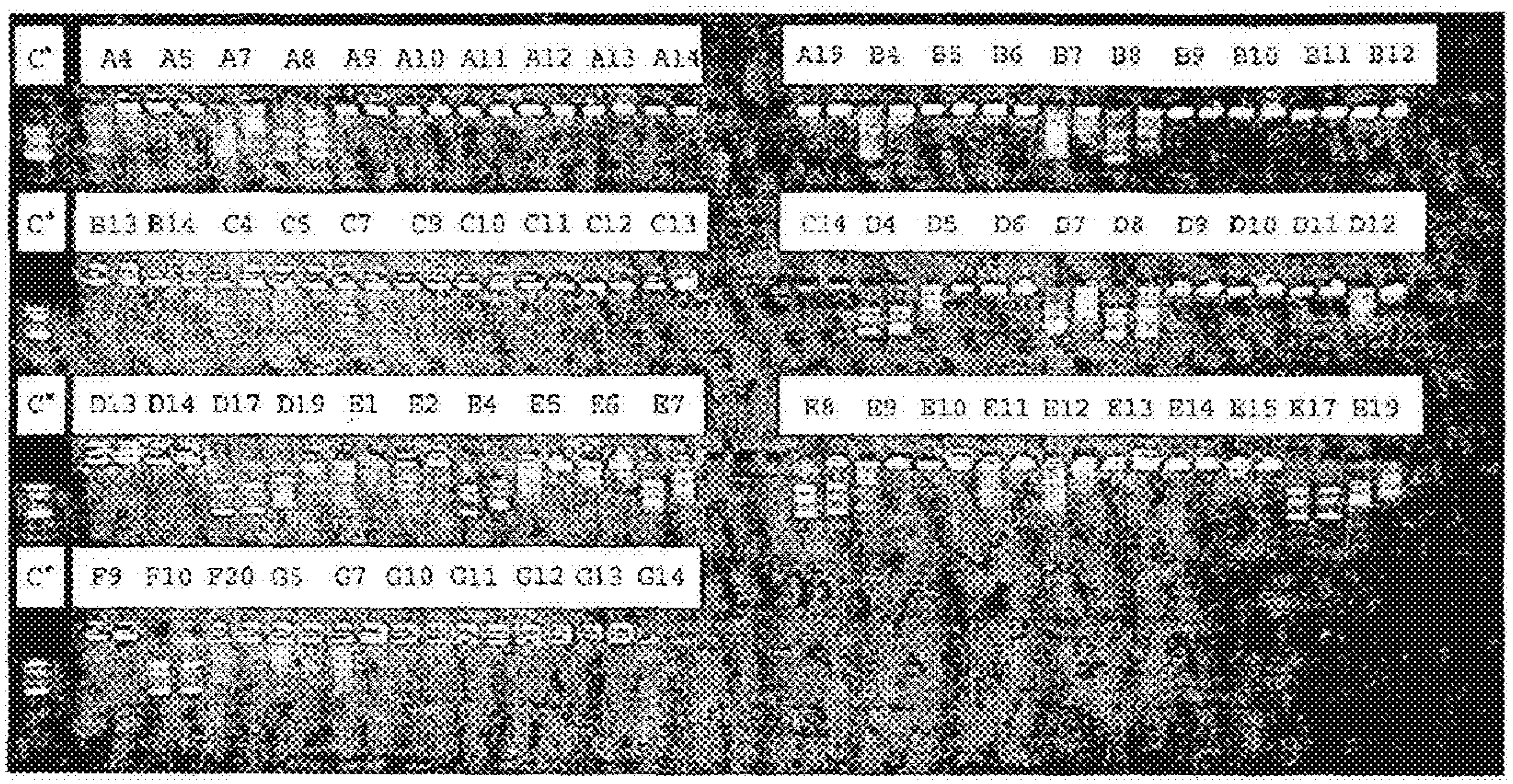

C+
A4 A5 A7 A8 A9 A10 A11 A12 A13 A14
A19 B4 B5 B6 B7 B8 B9 B10 B11 B12
C+
B13 B14 C4 C5 C7 C9 C10 C11 C12 C13
C14 D4 D5 D6 D7 D8 D9 D10 D11 D12
C+
D13 D14 D17 D19 E1 E2 E4 E5 E6 E7
E8 E9 E10 E11 E12 E13 E14 E15 E17 E19
C+
F9 F10 F20 G5 G7 G10 G11 G12 G13 G14

Figure 9

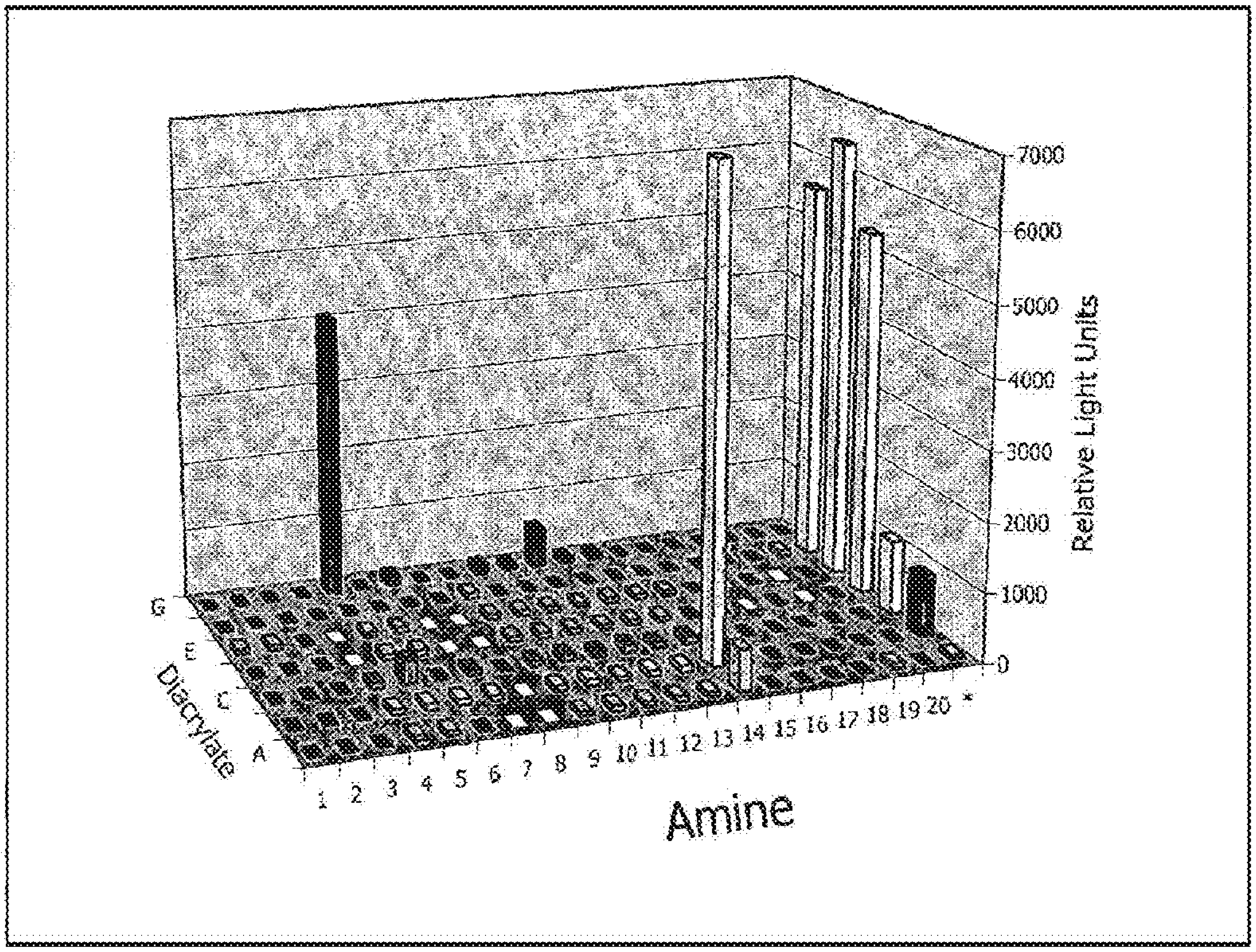

7000
6000
5000
4000
3000
2000
1000
0
Relative Light Units
G
E
C
A
Diacrylate
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 *
Amine

Figure 10

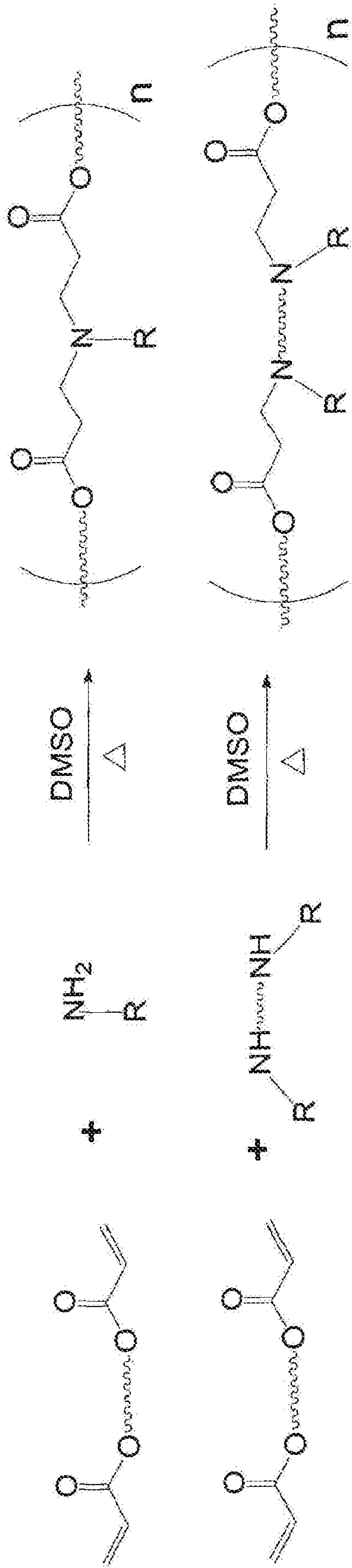



Figure 11

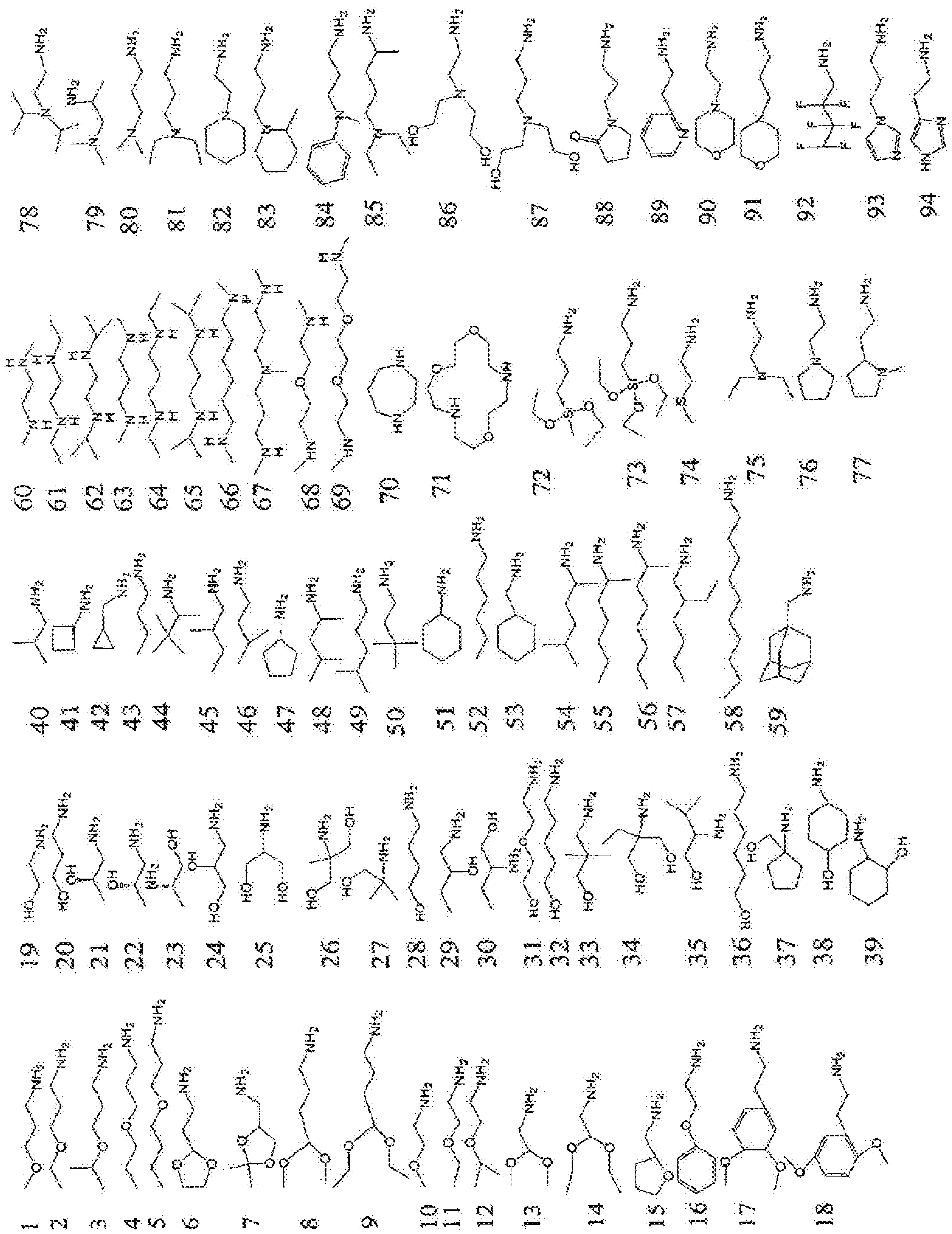


Figure 12

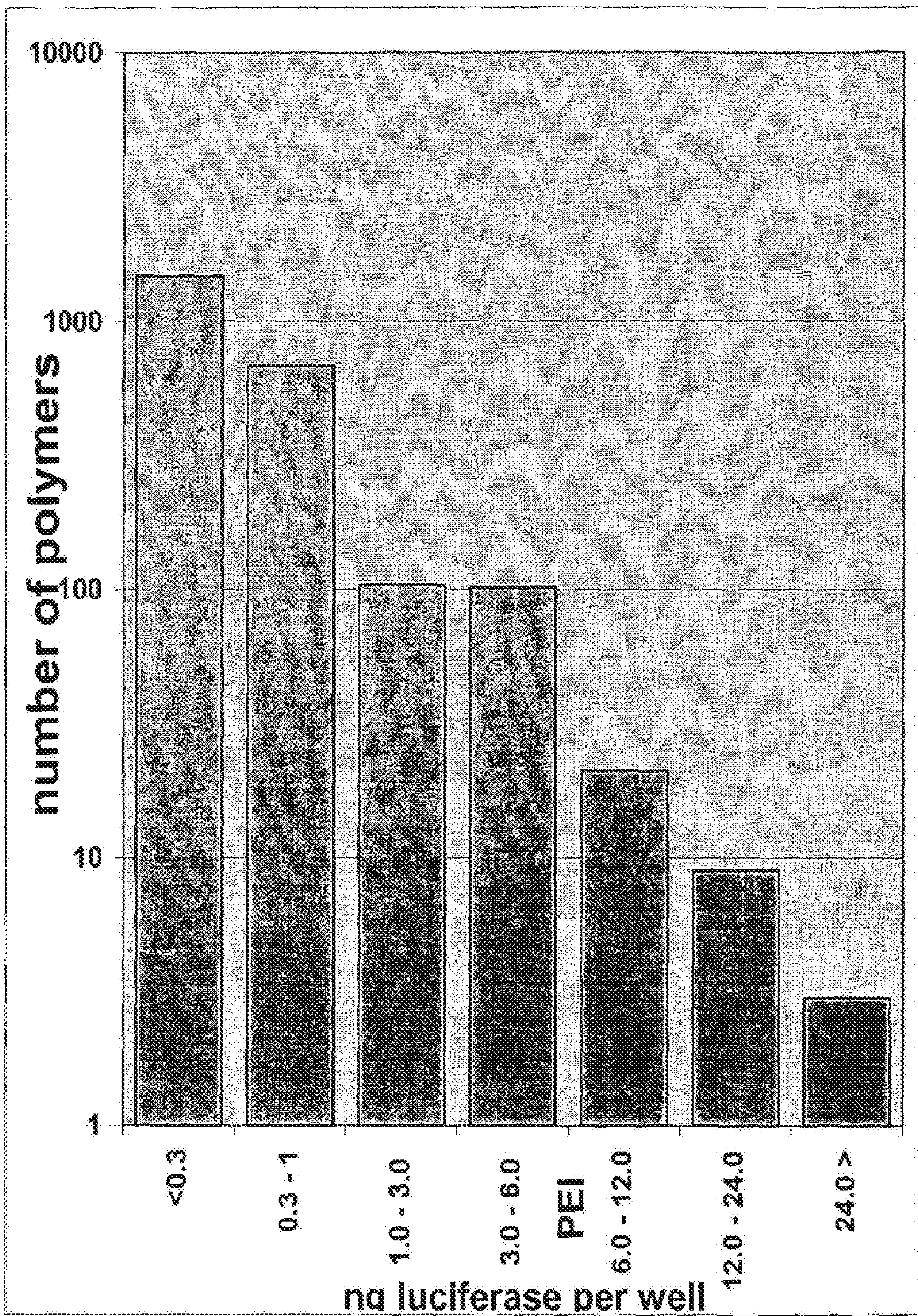

10000
1000
100
10
1
number of polymers
<0.3
0.3 - 1
1.0 - 3.0
3.0 - 6.0
PEI
6.0 - 12.0
12.0 - 24.0
24.0 >
ng luciferase per well

Figure 13

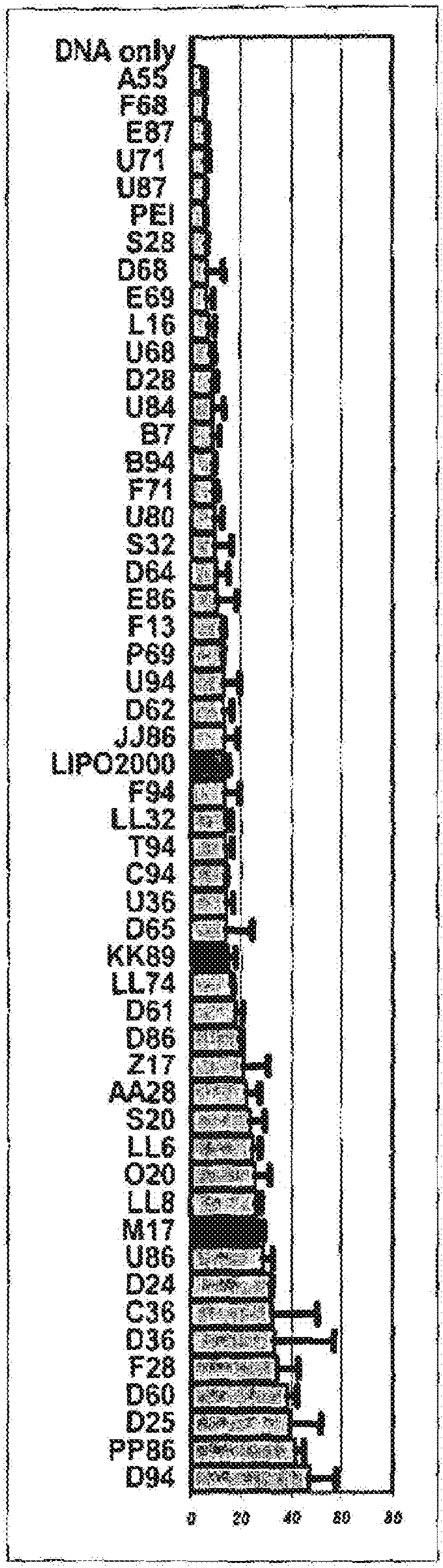
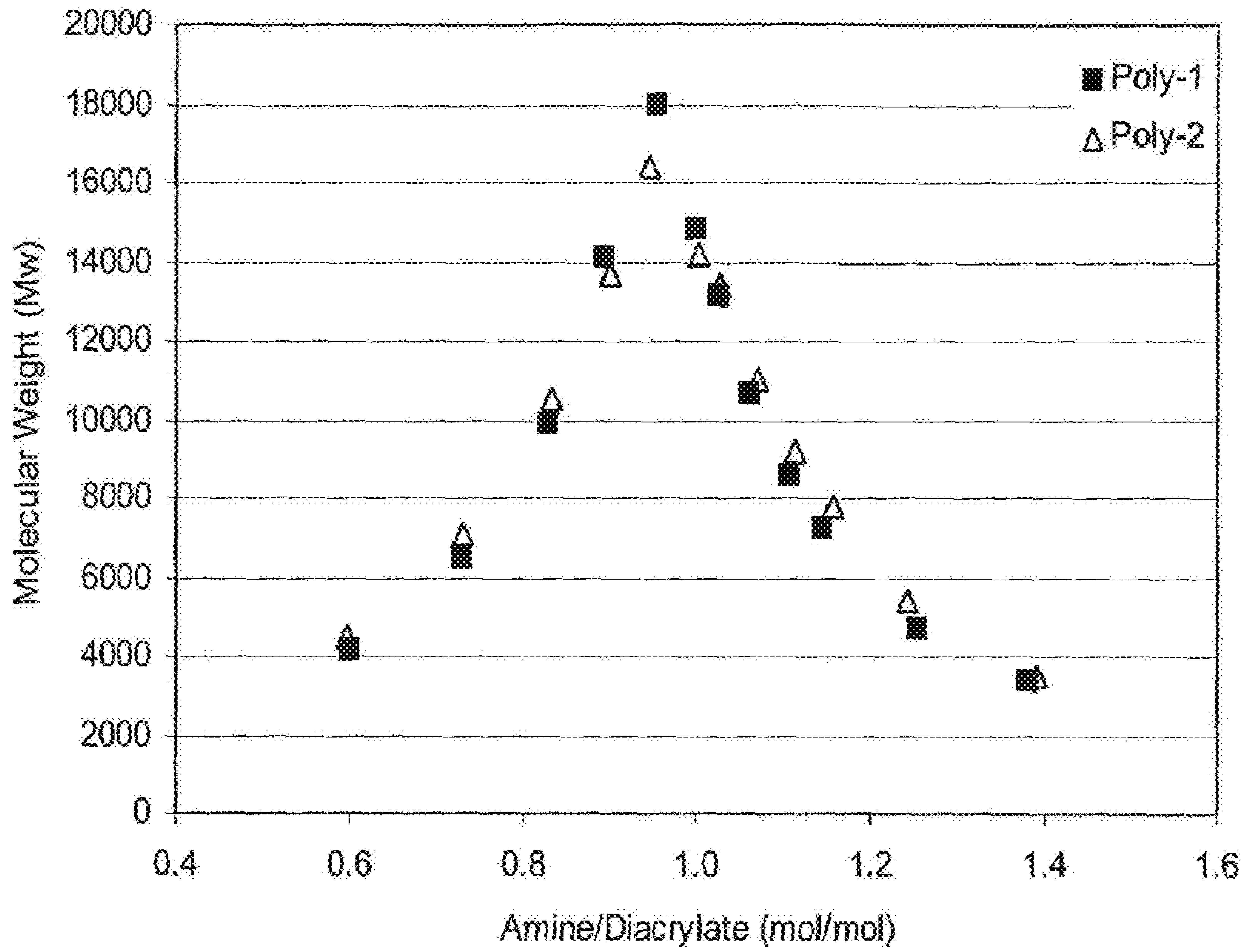

DNA only
A55
F68
E87
U71
U87
PEI
S28
D68
E69
L16
U68
D28
U84
B7
B94
F71
U80
S32
D64
E86
F13
P69
U94
D62
JJ86
LIPO2000
F94
LL32
T94
C94
U36
D65
KK89
LL74
D61
D86
Z17
AA28
S20
LL6
O20
LL8
M17
U86
D24
C36
D36
F28
D60
D25
PP86
D94
0
20
40
60
80

Figure 14

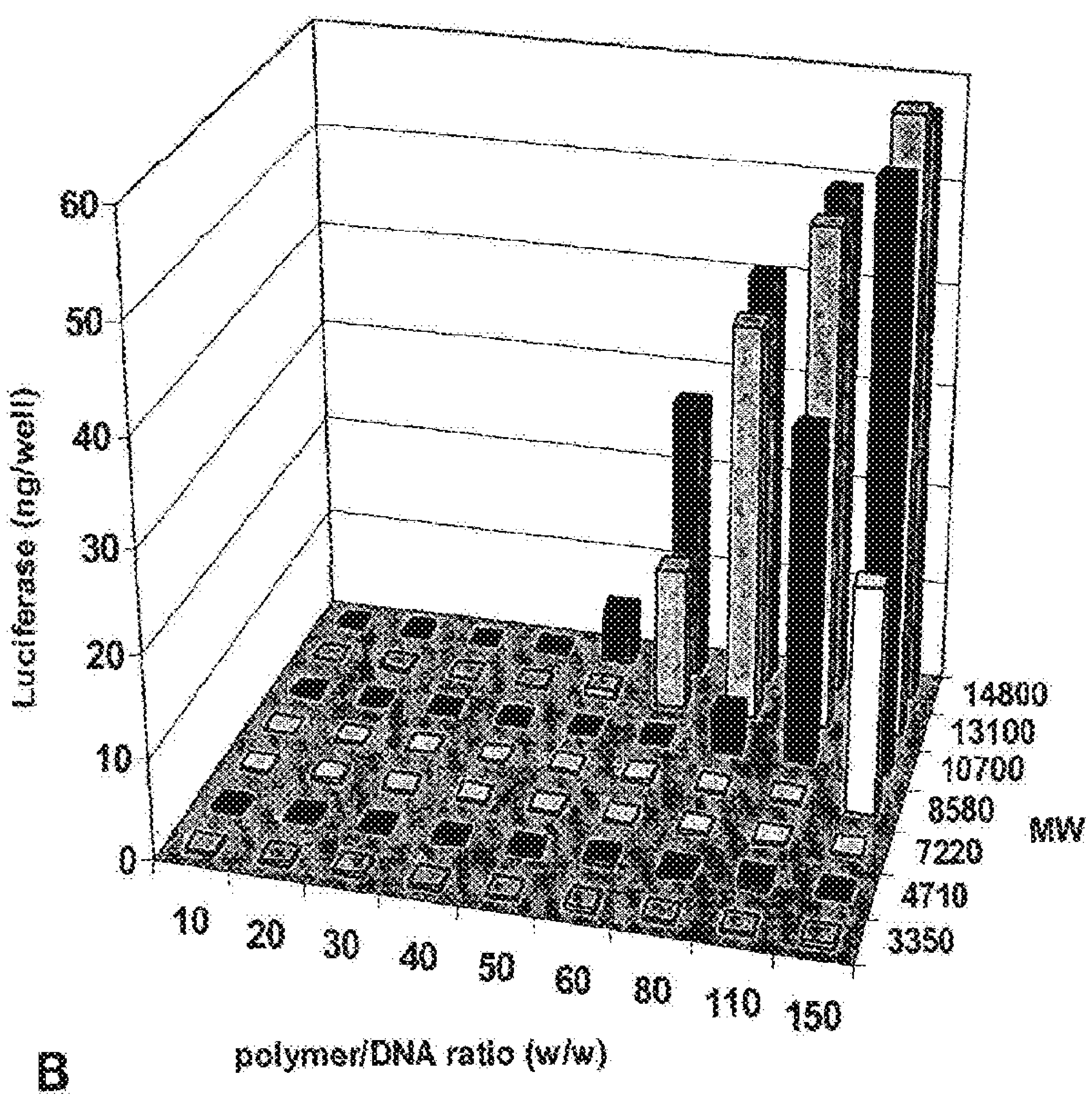
| Polymer | Binding |
|---|---|
| A55 | + |
| F68 | + |
| E87 | + |
| U71 | + |
| U87 | + |
| S28 | + |
| D68 | + |
| E69 | + |
| L16 | (+/-) |
| U68 | + |
| D28 | + |
| U84 | + |
| B7 | (+/-) |
| F71 | + |
| U80 | + |
| S32 | + |
| D64 | + |
| E86 | + |
| F13 | (+/-) |
| P69 | + |
| U94 | + |
| D62 | + |
| JJ86 | + |
| F94 | + |
| LL32 | + |
| T94 | + |
| C94 | + |
| U36 | + |
| D65 | + |
| KK89 | - |
| LL74 | + |
| D61 | + |
| D86 | + |
| Z17 | + |
| AA28 | + |
| S20 | + |
| LL6 | + |
| O20 | + |
| LL8 | + |
| M17 | - |
| U86 | + |
| D24 | + |
| C36 | + |
| D36 | + |
| F28 | + |
| D60 | + |
| D25 | + |
| PP86 | + |
| D94 | + |

Figure 15



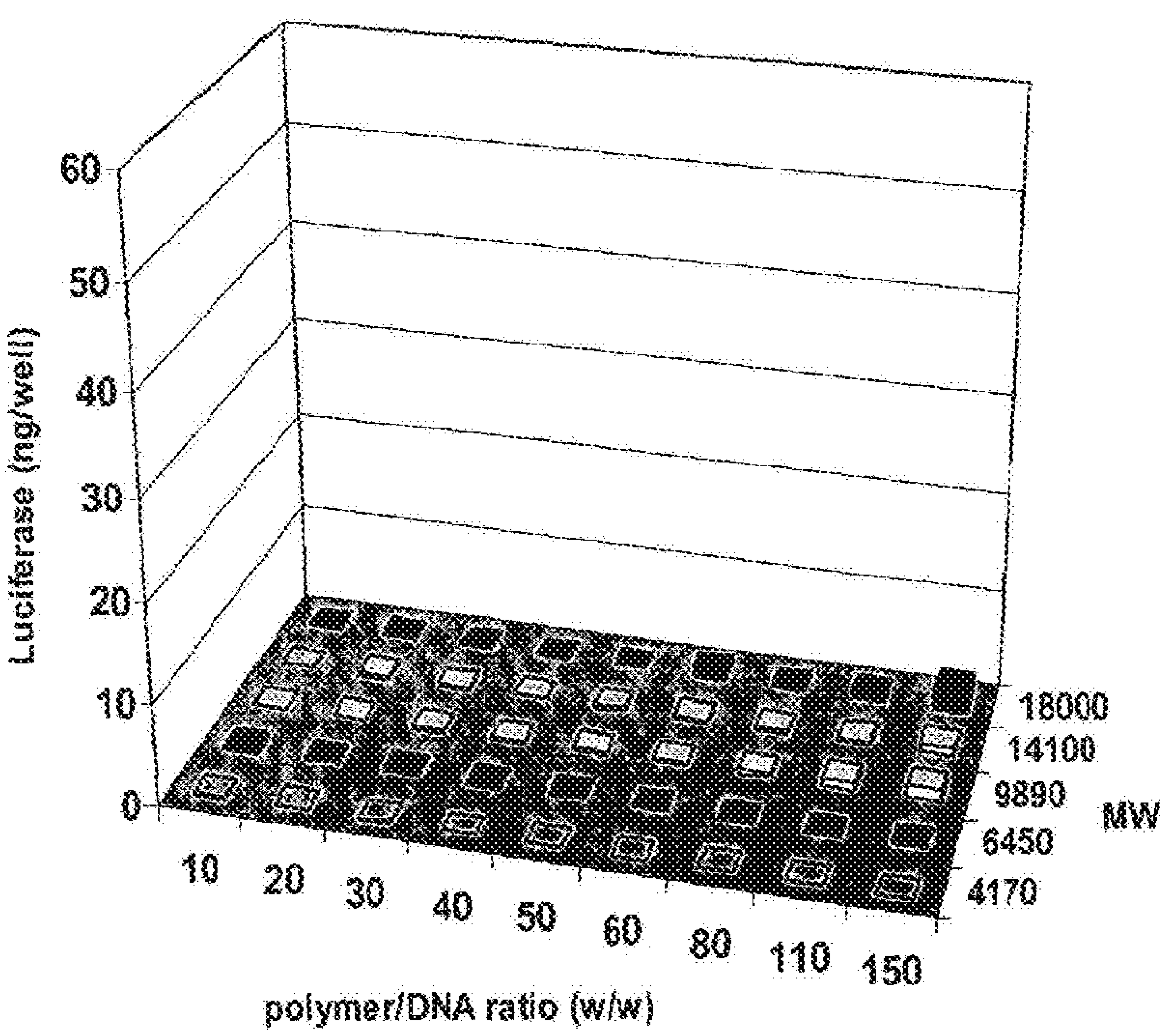
Poly-1
Poly-2
Molecular Weight (Mw)
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Amine/Diacrylate (mol/mol)


Figure 16



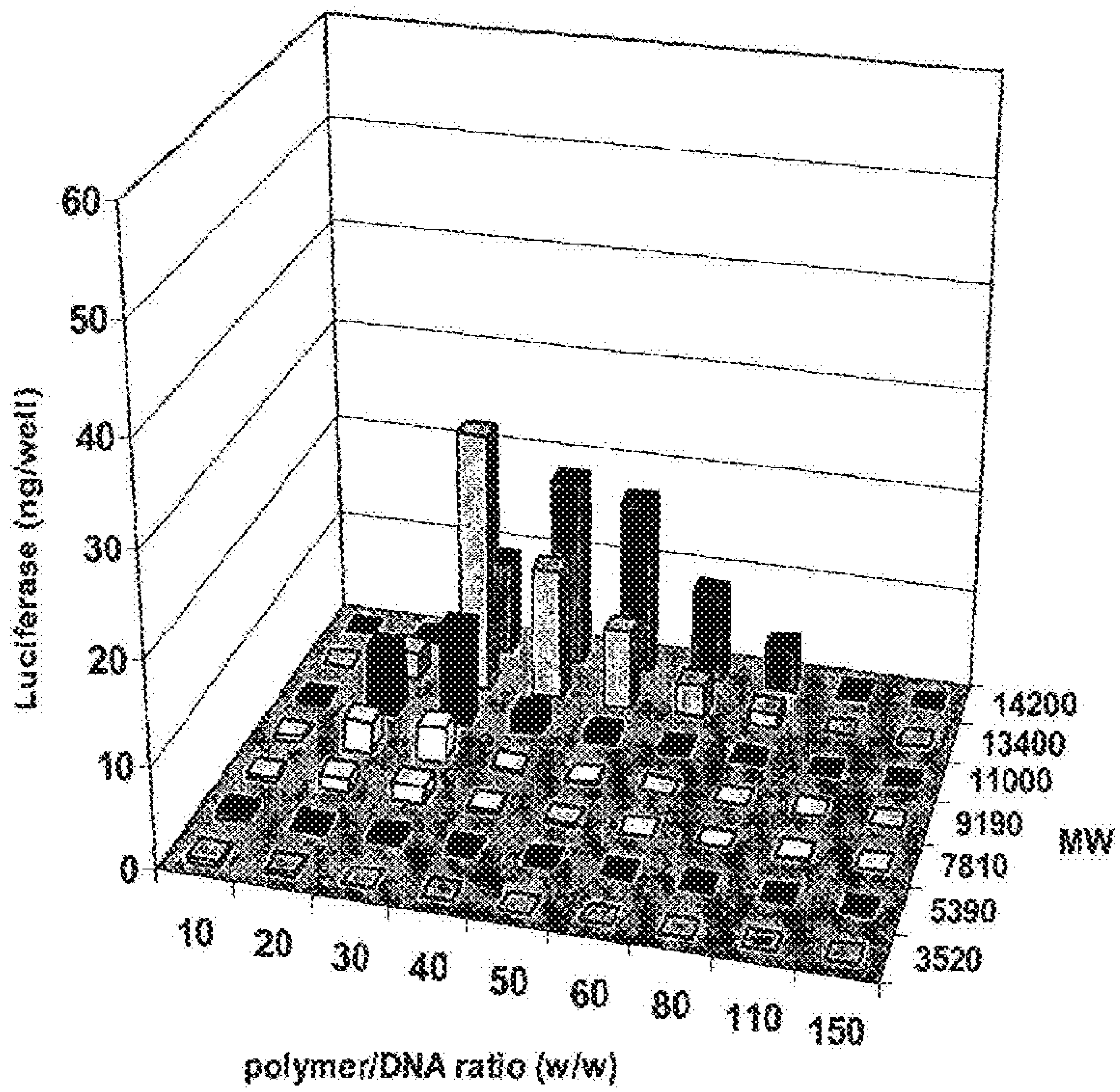
Luciferase (ng/well)
60
50
40
30
20
10
0
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
14800
13100
10700
8580
7220
4710
3350
MW


B



Luciferase (ng/well)
60
50
40
30
20
10
0
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
18000
14100
9890
6450
4170
MW


Figure 17


60
50
40
30
20
10
0
Luciferase (ng/well)
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
14200
13400
11000
9190
7810
5390
3520
MW

B

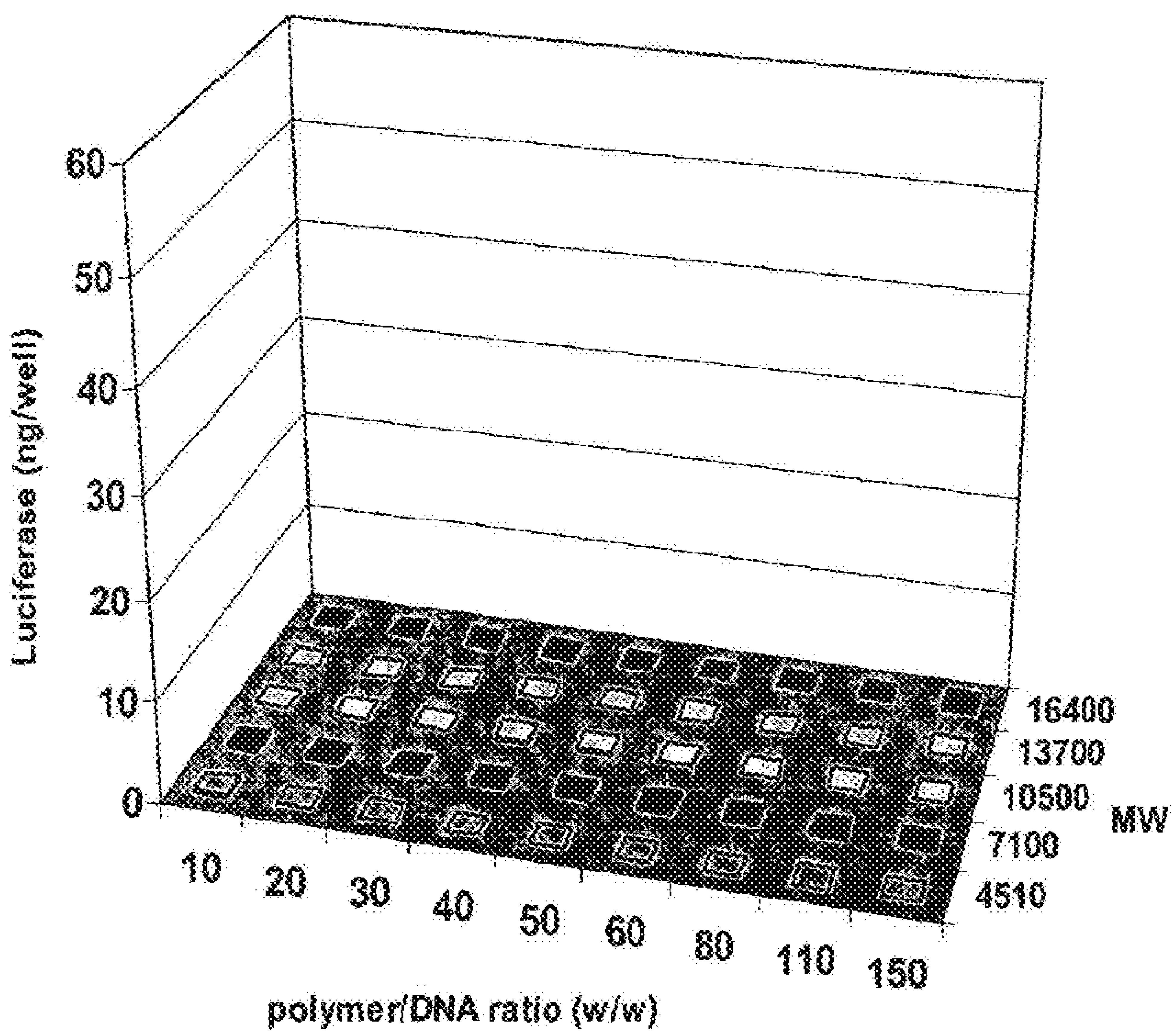
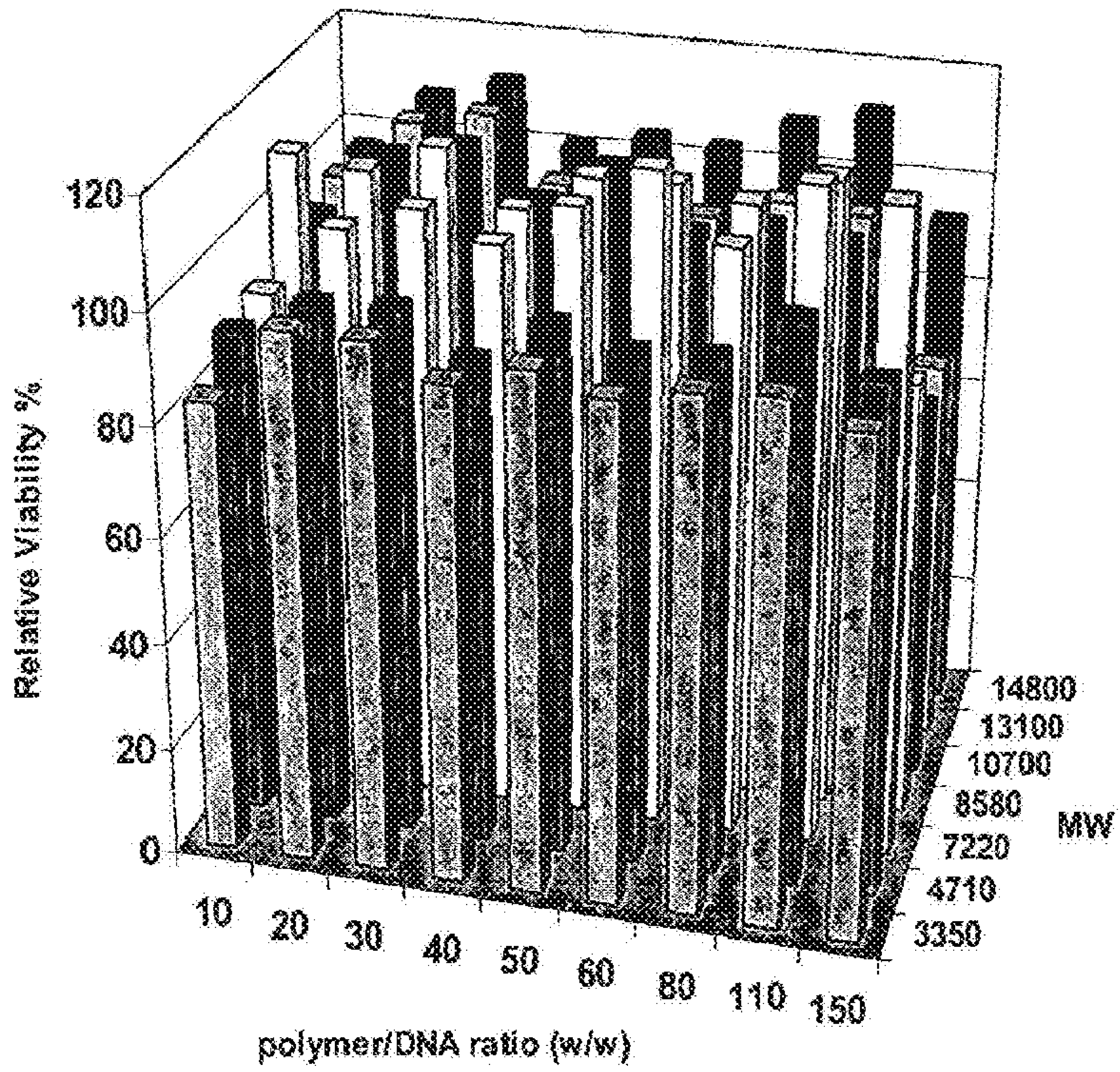
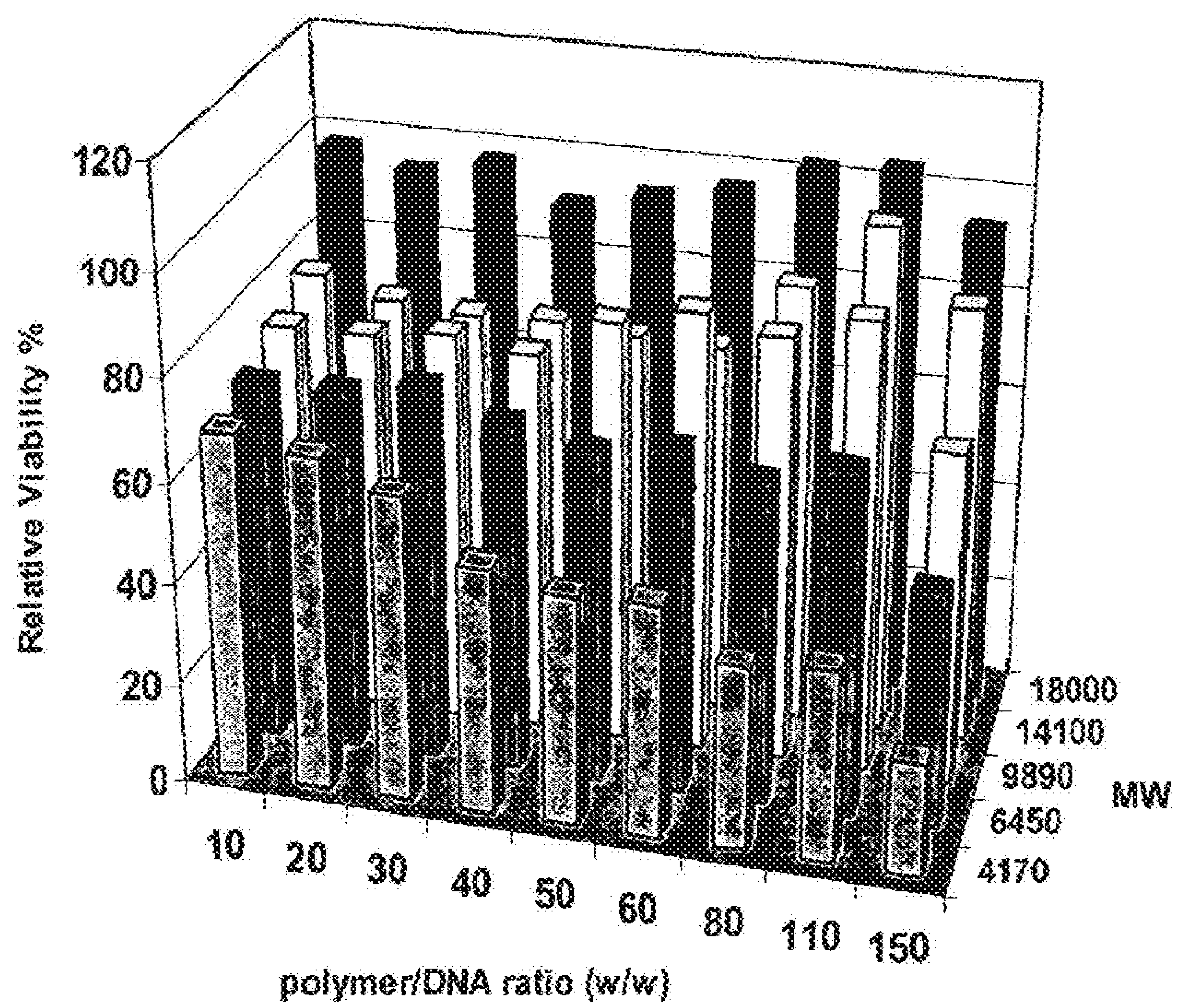

60
50
40
30
20
10
0
Luciferase (ng/well)
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
16400
13700
10500
7100
4510
MW

Figure 18

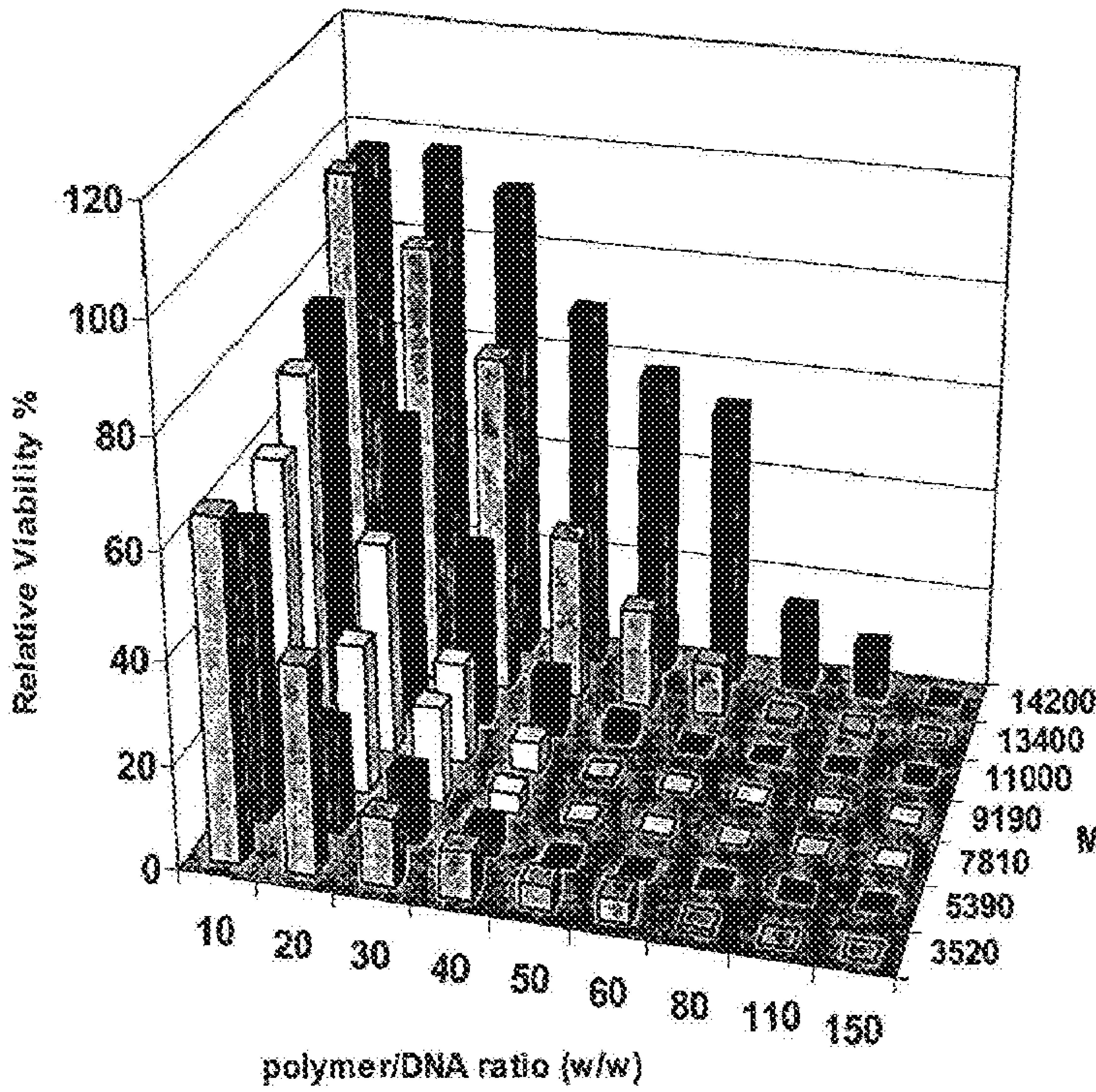


Relative Viability %
0
20
40
60
80
100
120
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
14800
13100
10700
8580
7220
4710
3350
MW

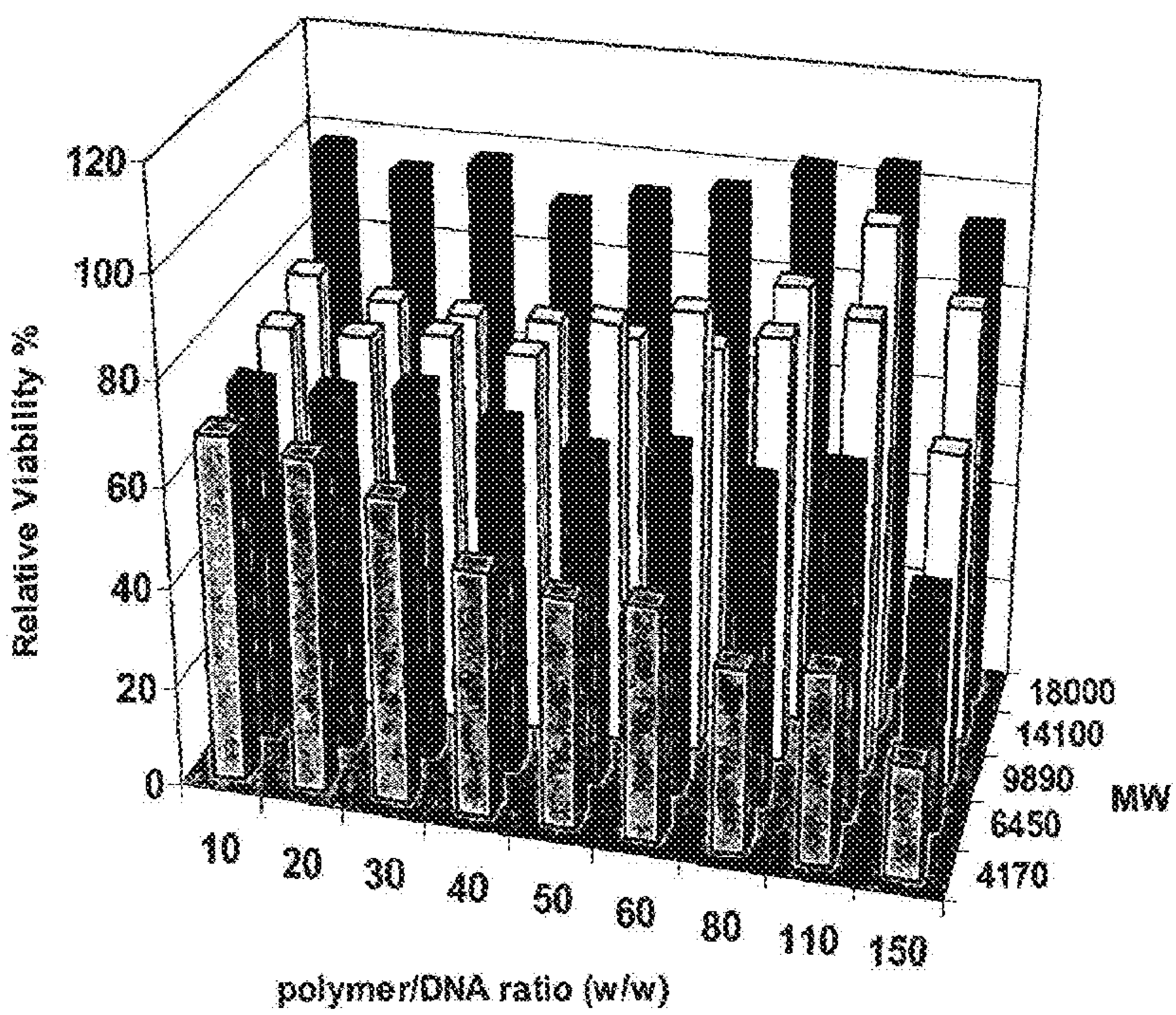



Relative Viability %
0
20
40
60
80
100
120
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
18000
14100
9890
6450
4170
MW


Figure 19



Relative Viability %
120
100
80
60
40
20
0
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
14200
13400
11000
9190
7810
5390
3520
MW

Relative Viability %
120
100
80
60
40
20
0
10
20
30
40
50
60
80
110
150
polymer/DNA ratio (w/w)
18000
14100
9890
6450
4170
MW


Figure 20

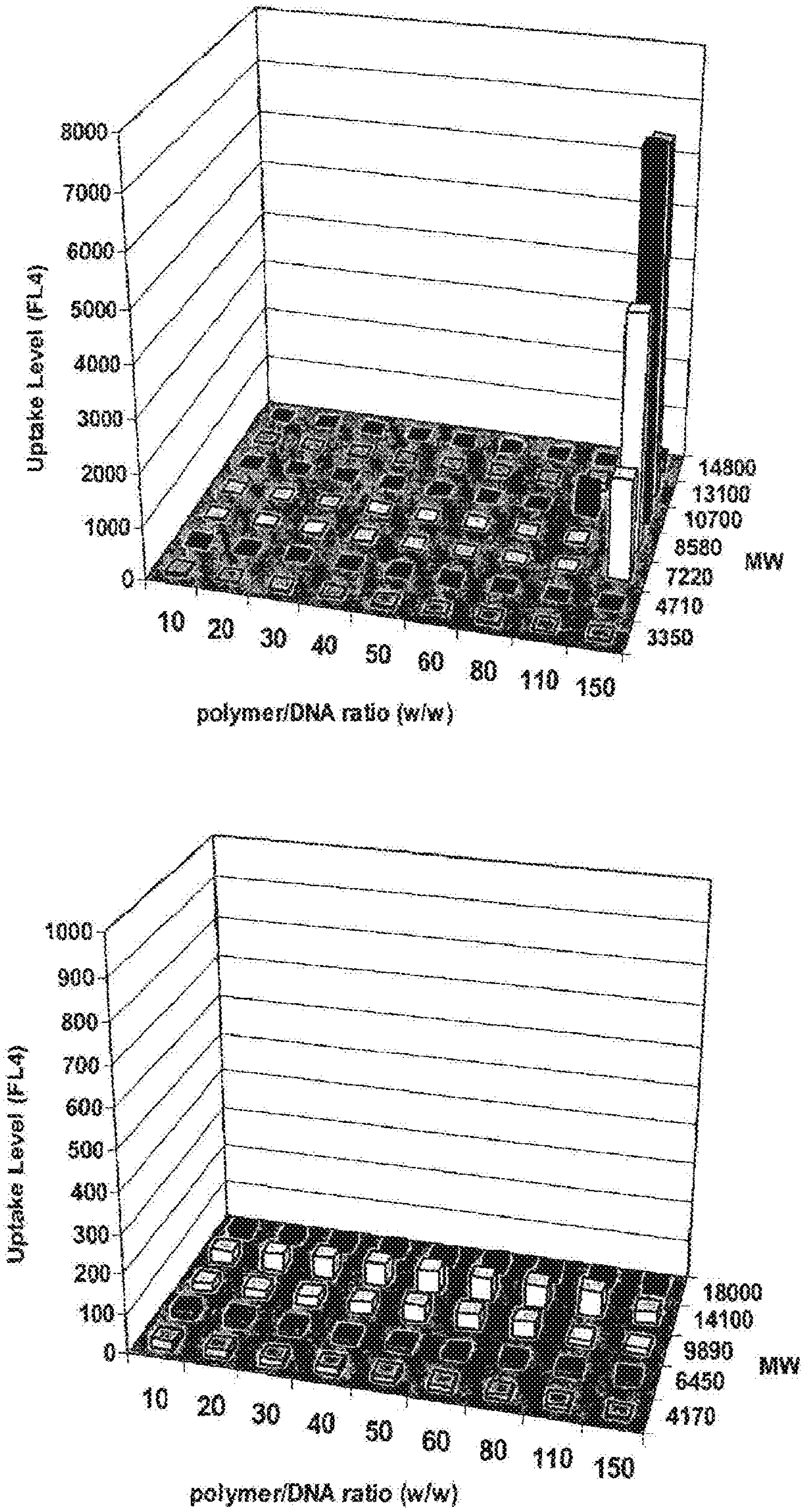

Uptake Level (FL4)
8000
7000
6000
5000
4000
3000
2000
1000
0
10 20 30 40 50 60 80 110 150
polymer/DNA ratio (w/w)
14800
13100
10700
8580
7220
4710
3350
MW
Uptake Level (FL4)
1000
900
800
700
600
500
400
300
200
100
0
10 20 30 40 50 60 80 110 150
polymer/DNA ratio (w/w)
18000
14100
9890
6450
4170
MW

Figure 21

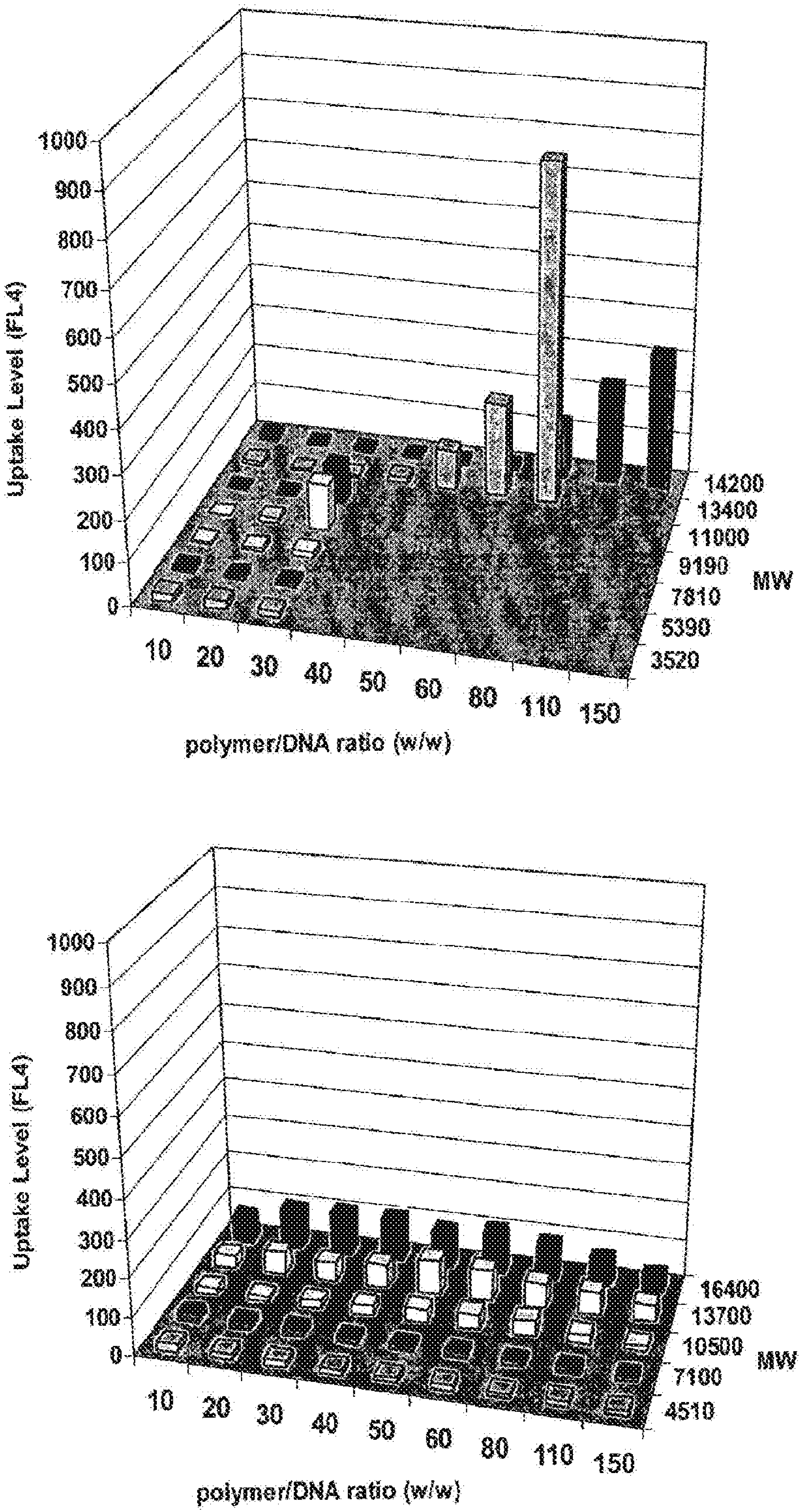
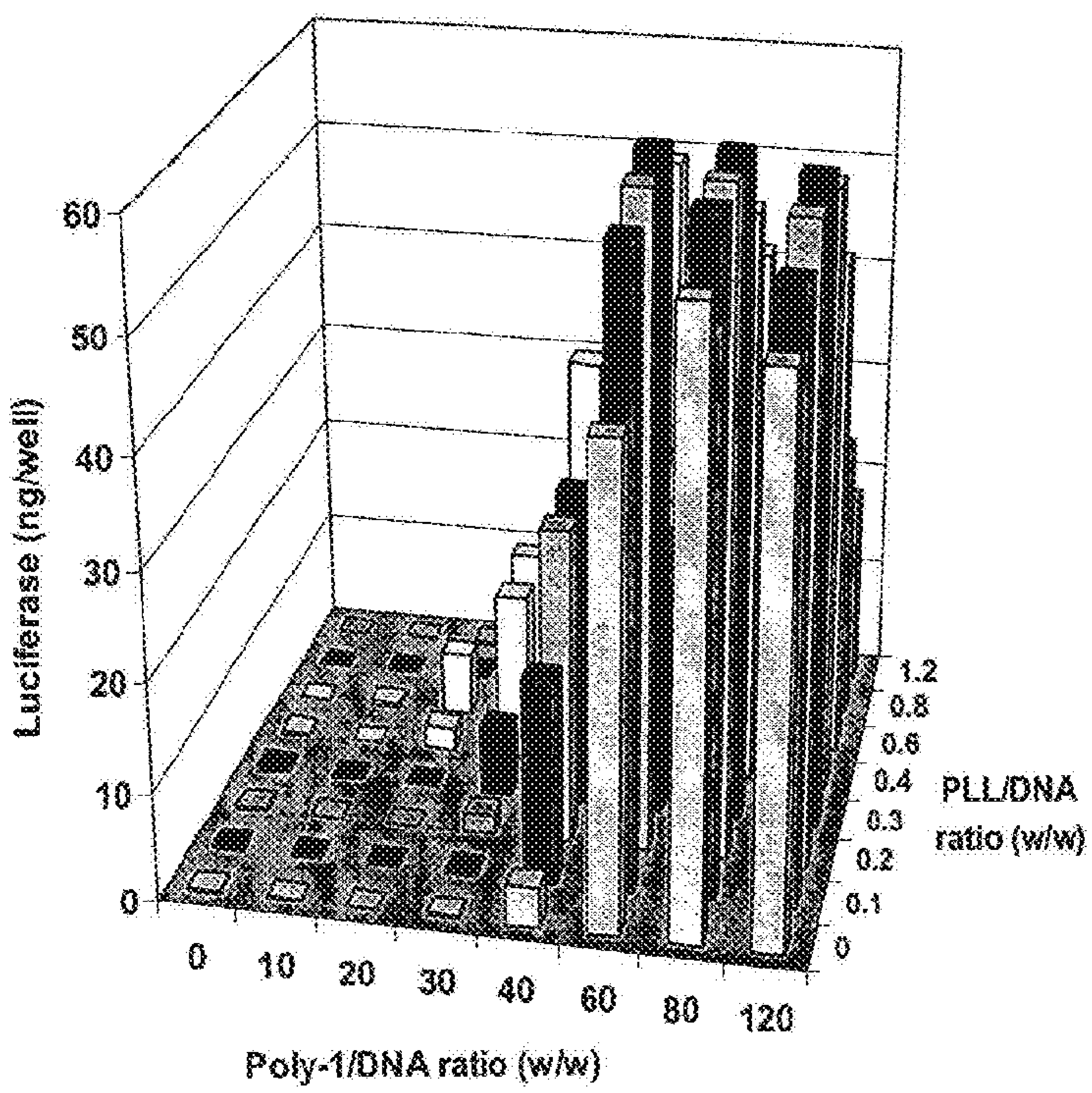

Uptake Level (FL4)
1000
900
800
700
600
500
400
300
200
100
0
10 20 30 40 50 60 80 110 150
polymer/DNA ratio (w/w)
14200
13400
11000
9190
7810
5390
3520
MW
Uptake Level (FL4)
1000
900
800
700
600
500
400
300
200
100
0
10 20 30 40 50 60 80 110 150
polymer/DNA ratio (w/w)
16400
13700
10500
7100
4510
MW

Figure 22


60
50
40
30
20
10
0
Luciferase (ng/well)
0
10
20
30
40
60
80
120
Poly-1/DNA ratio (w/w)
1.2
0.8
0.6
0.4
0.3
0.2
0.1
0
PLL/DNA
ratio (w/w)

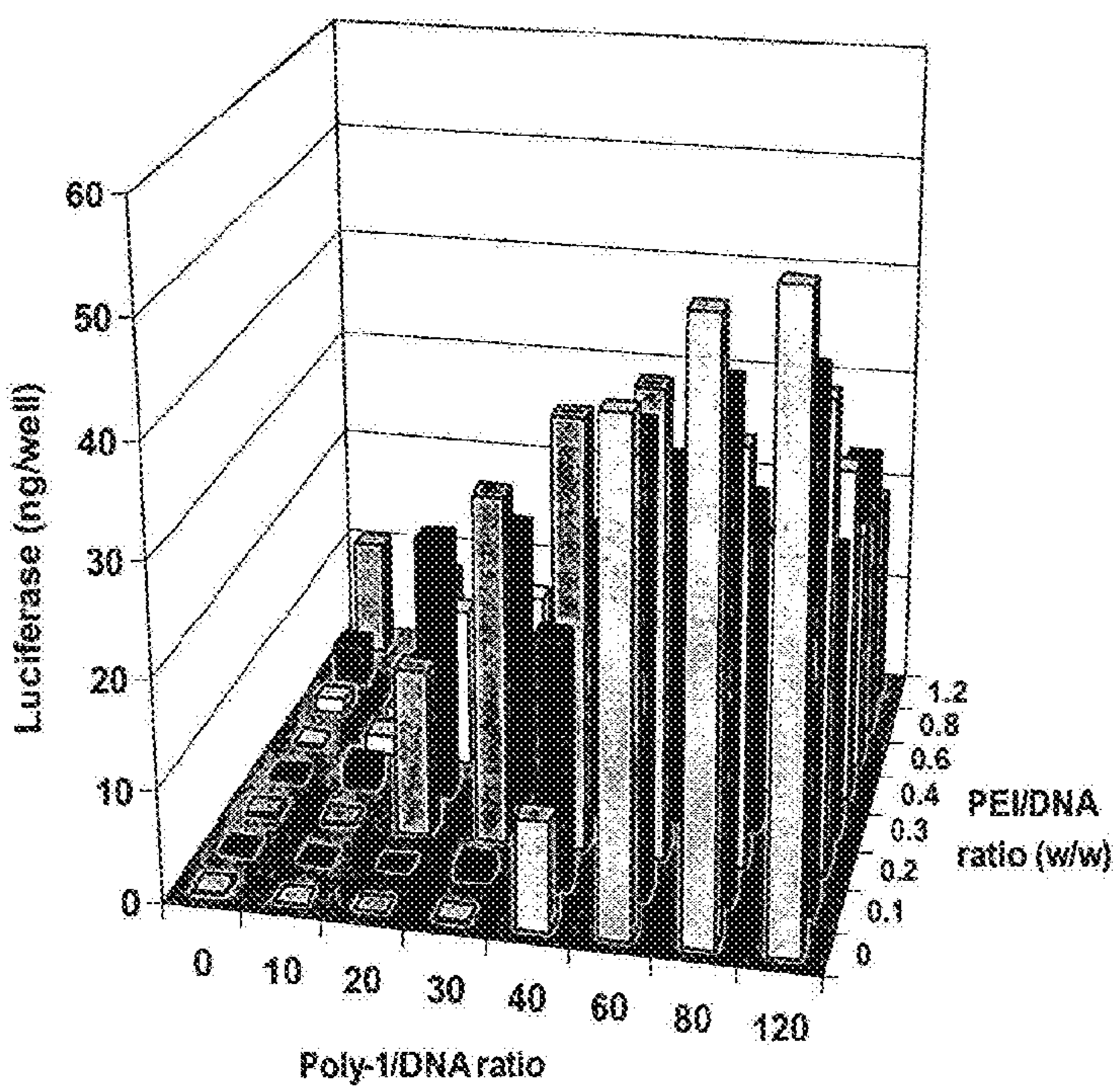
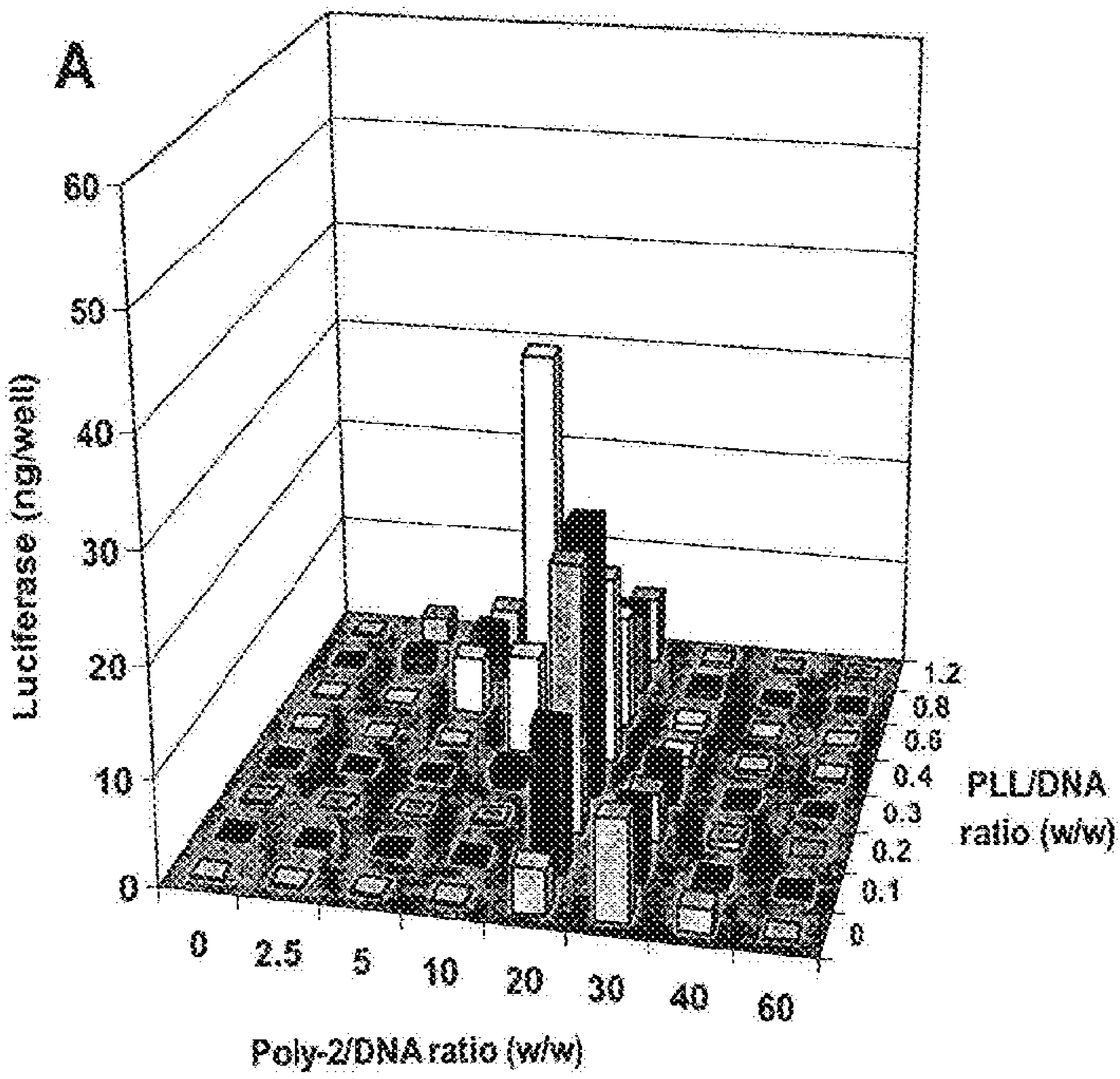

60
50
40
30
20
10
0
Luciferase (ng/well)
0
10
20
30
40
60
80
120
Poly-1/DNA ratio
1.2
0.8
0.6
0.4
0.3
0.2
0.1
0
PEI/DNA
ratio (w/w)

Figure 23


A
Luciferase (ng/well)
60
50
40
30
20
10
0
0
2.5
5
10
20
30
40
60
Poly-2/DNA ratio (w/w)
1.2
0.8
0.6
0.4
0.3
0.2
0.1
0
PLL/DNA ratio (w/w)

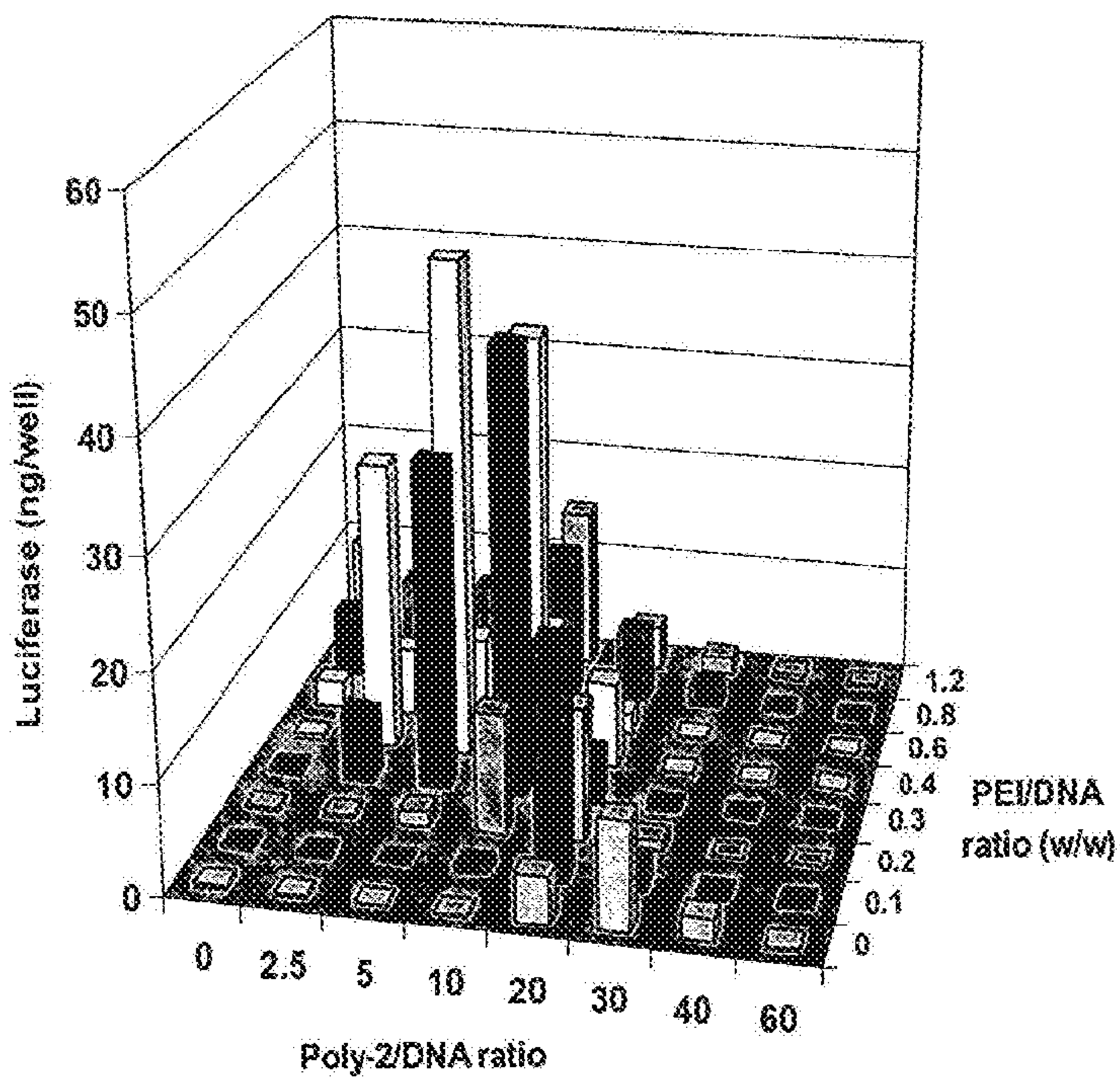

B
Luciferase (ng/well)
60
50
40
30
20
10
0
0
2.5
5
10
20
30
40
60
Poly-2/DNA ratio
1.2
0.8
0.6
0.4
0.3
0.2
0.1
0
PEI/DNA ratio (w/w)

Figure 24

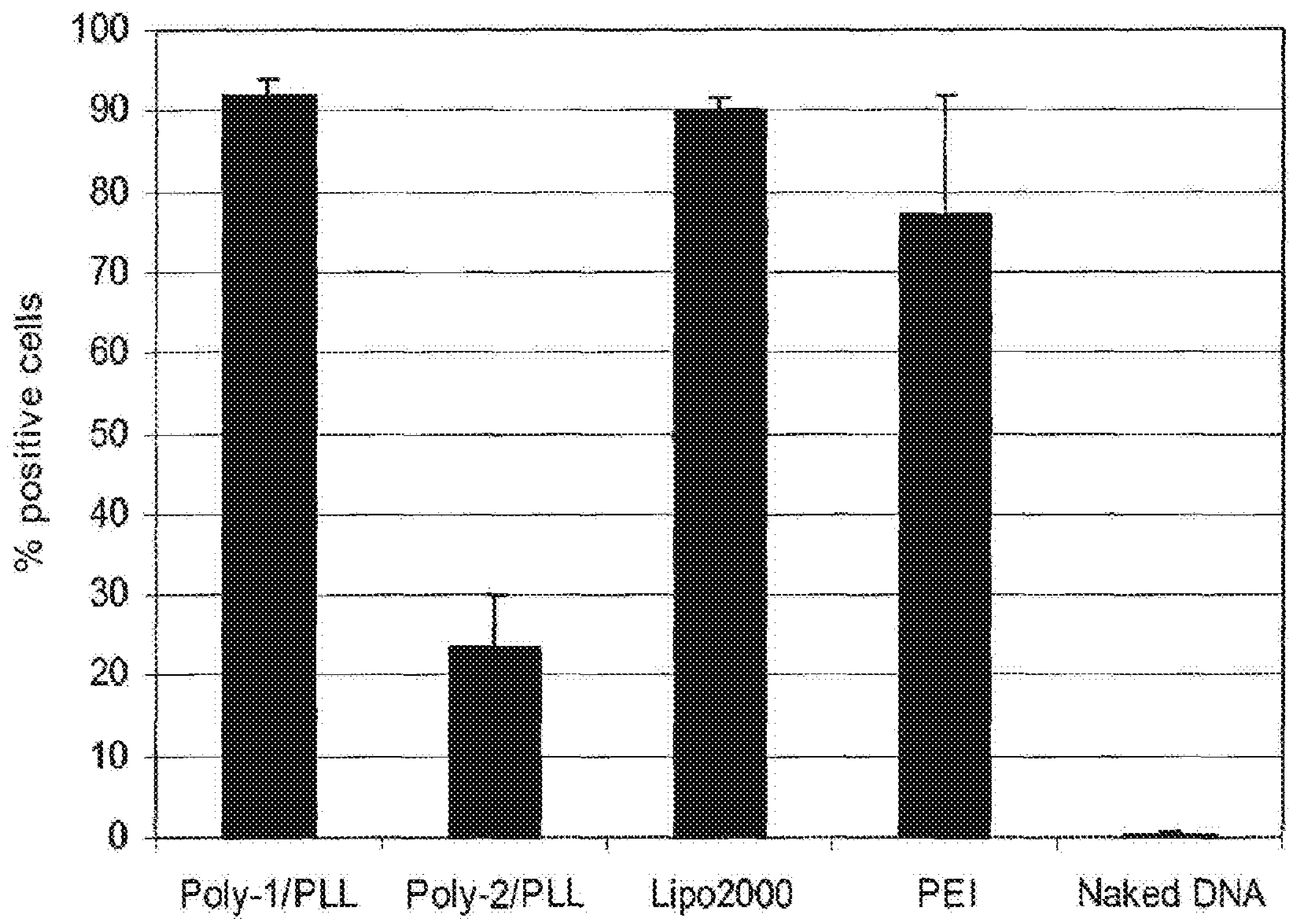

100
90
80
70
60
50
40
30
20
10
0
% positive cells
Poly-1/PLL
Poly-2/PLL
Lipo2000
PEI
Naked DNA

Figure 25

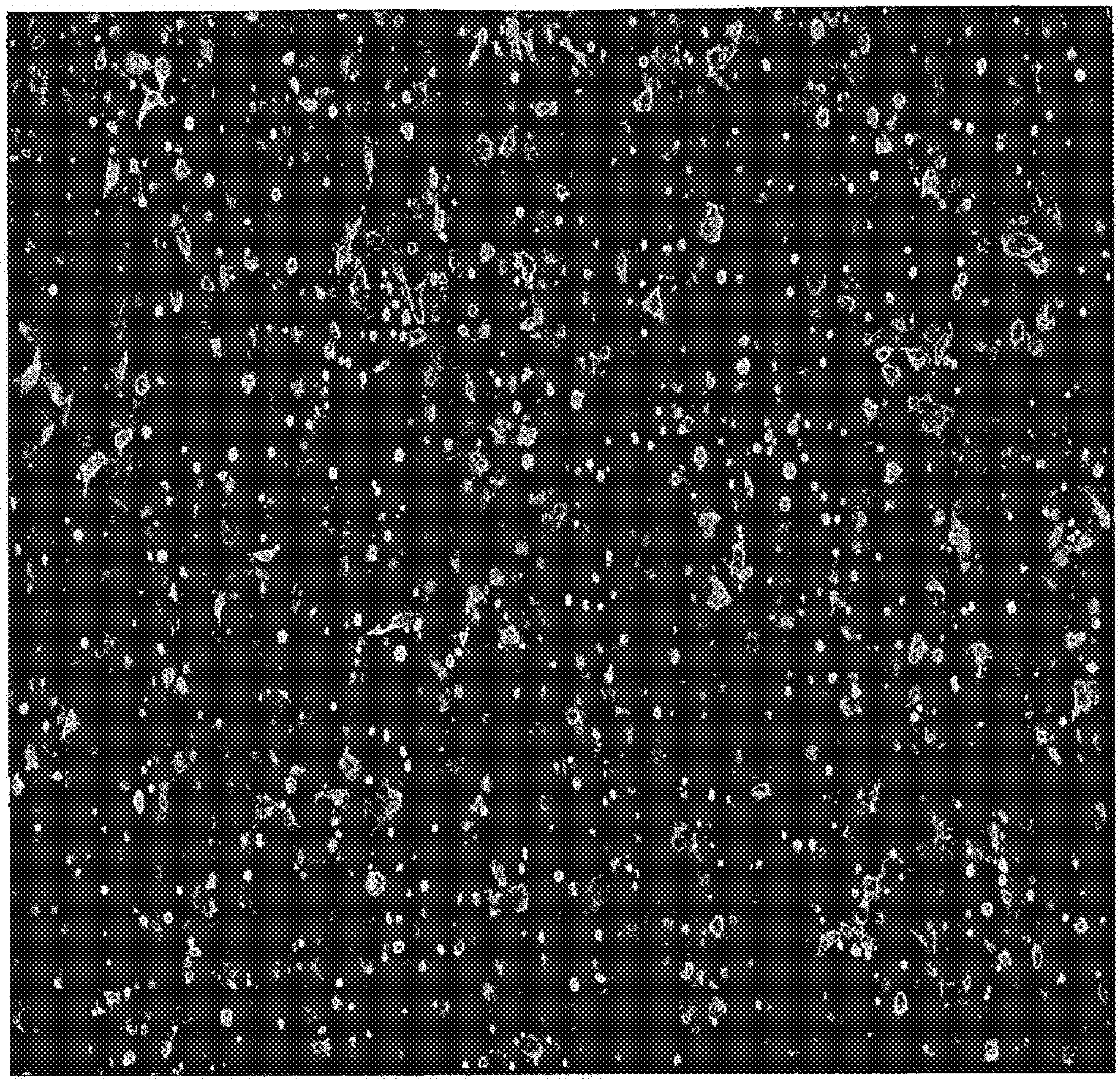


Figure 26

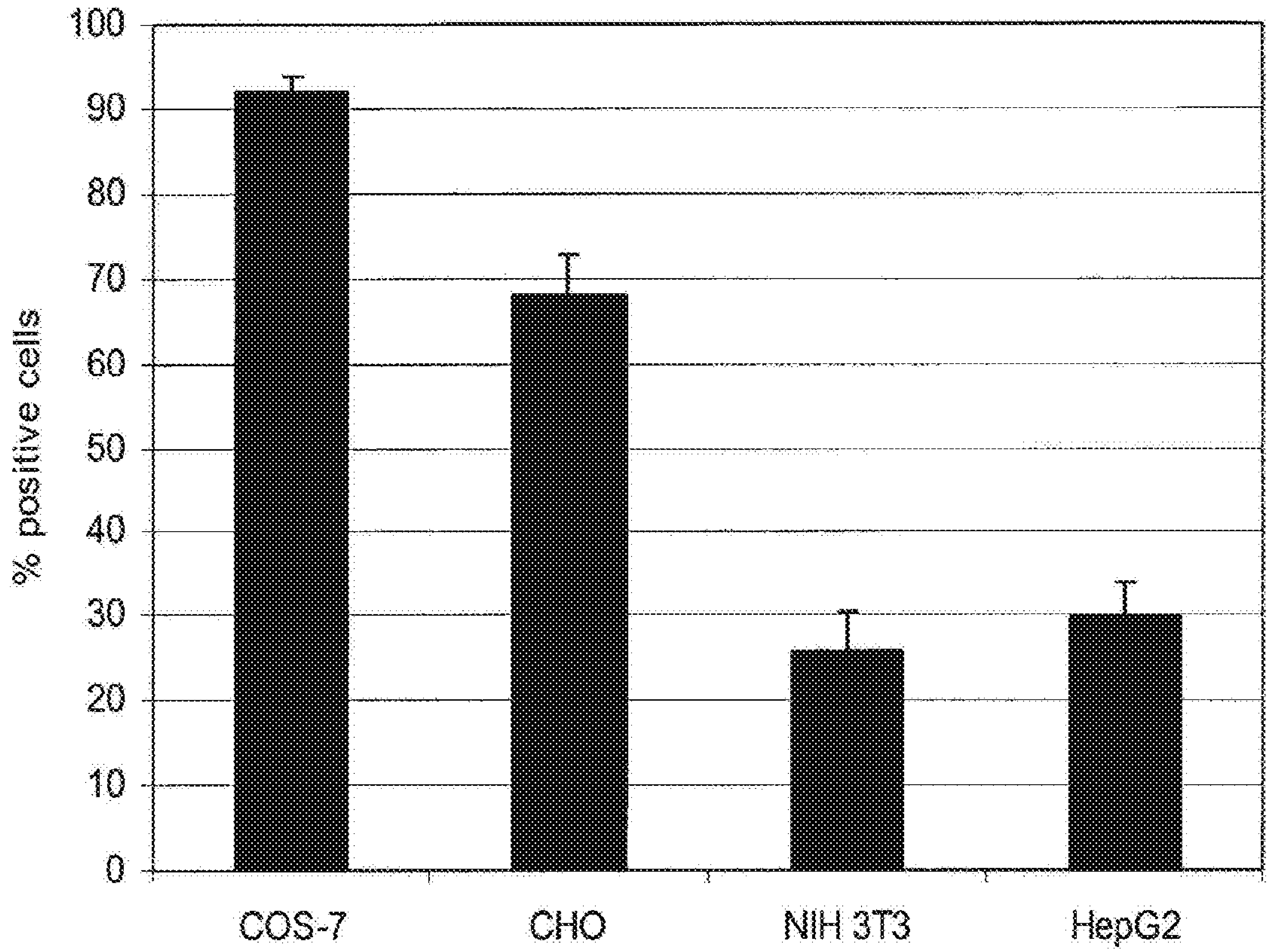

100
90
80
70
60
50
40
30
20
10
0
% positive cells
COS-7
CHO
NIH 3T3
HepG2

Figure 27

## BIODEGRADABLE POLY(β-AMINO ESTERS) AND USES THEREOF

**Matter enclosed in heavy brackets [ ] appears in the original patent but forms no part of this reissue specification; matter printed in italics indicates the additions made by reissue.**

### RELATED APPLICATIONS

*More than one reissue application has been filed for the reissue of U.S. Pat. No. 7,427,394. The reissue applications are U.S. patent applications, U.S. Ser. No. 12/568,481 (the present application), filed Sep. 28, 2009, and U.S. Ser. No. 12/833,749, filed Jul. 9, 2010, which is a divisional of U.S. Ser. No. 12/568,481.*

The present application, *U.S. patent application, U.S. Ser. No. 12/568,481, filed Sep. 28, 2009, is a reissue application of and claims priority under 35 U.S.C. §120 to U.S. patent application, U.S. Ser. No. 10/446,444, filed May 28, 2003, issued as U.S. Pat. No. 7,427,394, which* claims priority under 35 U.S.C. §120 to and is a continuation-in-part of application U.S. Ser. No. 09/969,431, filed Oct. 2, 2001, now U.S. Pat. No. 6,998,115 entitled "Biodegradable Poly(beta-amino esters) and Uses Thereof," which claims priority to provisional applications, U.S. Ser. No. 60/305,337, filed Jul. 13, 2001, and U.S. Ser. No. 60/239,330, filed Oct. 10, 2000, each of which is incorporated herein by reference.

### GOVERNMENT SUPPORT

**[**The work described herein was supported, in part, by grants from the National Science Foundation (Cooperative Agreement #ECC9843342 to the MIT Biotechnology Process Engineering Center), the National Institutes of Health (GM26698; NRSA Fellowship #1 F32 GM20227-01), and the Department of the Army (Cooperative Agreement # DAMD 17-99-2-9-001 to the Center for Innovative Minimally Invasive Therapy). The United States government may have certain rights in the invention.**]** *This invention was made with Government support under Grant No. DAMD 17-02-2-006, awarded by the U.S. Army and under Grant No. EEC9843342, awarded by the National Science Foundation. The Government has certain rights in this invention.*

### BACKGROUND OF THE INVENTION

The treatment of human diseases through the application of nucleotide-based drugs such as DNA and RNA has the potential to revolutionize the medical field (Anderson Nature 392 (Suppl.):25-30, 1996; Friedman Nature Med. 2:144-147, 1996; Crystal Science 270:404-410, 1995; Mulligan Science 260:926-932, 1993; each of which is incorporated herein by reference). Thus far, the use of modified viruses as gene transfer vectors has generally represented the most clinically successful approach to gene therapy. While viral vectors are currently the most efficient gene transfer agents, concerns surrounding the overall safety of viral vectors, which include the potential for unsolicited immune responses, have resulted in parallel efforts to develop non-viral alternatives (for leading references, see: Luo et al. Nat. Biotechnol. 18:33-37, 2000; Behr Acc. Chem. Res. 26:274-278, 1993; each of which is incorporated herein by reference). Current alternatives to viral vectors include polymeric delivery systems (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; each of which is



incorporated herein by reference), liposomal formulations (Miller Angew. Chem. Int. Ed. 37:1768-1785, 1998; Hope et al. Molecular Membrane Technology 15:1-14, 1998; Deshrnukh et al. New J. Chem. 21:113-124, 1997; each of which is incorporated herein by reference), and "naked" DNA injection protocols (Sanford Trends Biotechnol. 6:288-302, 1988; incorporated herein by reference). While these strategies have yet to achieve the clinical effectiveness of viral vectors, the potential safety, processing, and economic benefits offered by these methods (Anderson Nature 392(Suppl.):25-30, 1996; incorporated herein by reference) have ignited interest in the continued development of non-viral approaches to gene therapy (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Gonzalez et al. Bioconjugate Chem. 10:1068-1074, 1999; Kukowska-Latallo et al. Proc. Natl. Acad. Sci. USA 93:4897-4902, 1996; Tang et al. Bioconjugate Chem. 7:703-714, 1996; Haensler et al. Bioconjugate Chem. 4:372-379, 1993; each of which is incorporated herein by reference).

Cationic polymers have been widely used as transfection vectors due to the facility with which they condense and protect negatively charged strands of DNA. Amine-containing polymers such as poly(lysine) (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; each of which is incorporated herein by reference), poly(ethylene imine) (PEI) (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; incorporated herein by reference), and poly(amidoamine) dendrimers (Kukowska-Latallo et al. Proc. Natl. Acad. Sci. USA 93:4897-4902, 1996; Tang et al. Bioconjugate Chem. 7:703-714, 1996; Haensler et al. Bioconjugate Chem. 4:372-379, 1993; each of which is incorporated herein by reference) are positively-charged at physiological pH, form ion pairs with nucleic acids, and mediate transfection in a variety of cell lines. Despite their common use, however, cationic polymers such as poly(lysine) and PEI can be significantly cytotoxic (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Deshmukh et al. New J. Chem. 21:113-124, 1997; Choksakulnimitr et al. Controlled Release 34:233-241, 1995; Brazeau et al. Pharm. Res. 15:680-684, 1998; each of which is incorporated herein by reference). As a result, the choice of cationic polymer for a gene transfer application generally requires a trade-off between transfection efficiency and short- and long-term cytotoxicity. Additionally, the long-term biocompatibility of these polymers remains an important issue for use in therapeutic applications in vivo, since several of these polymers are not readily biodegradable (Uhrich Trends Polym. Sci. 5:388-393, 1997; Roberts et al. J. Biomed. Mater. Res. 30:53-65, 1996; each of which is incorporated herein by reference).

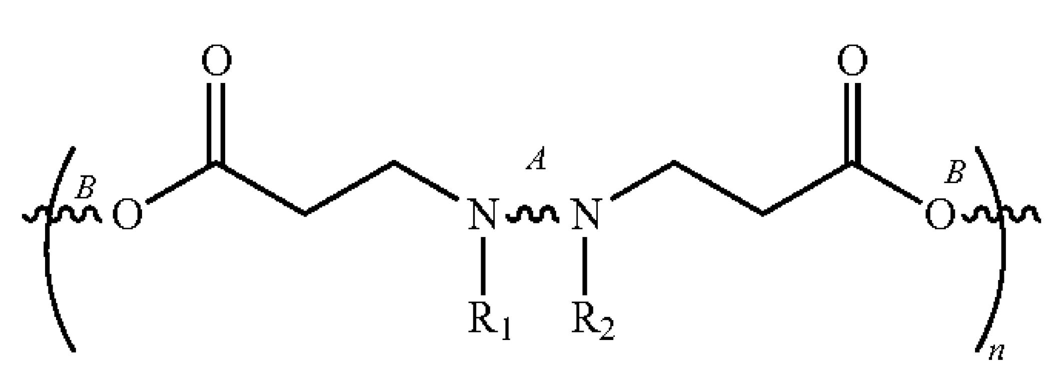
In order to develop safe alternatives to existing polymeric vectors and other functionalized biomaterials, degradable polyesters bearing cationic side chains have been developed (Putnam et al. Macromolecules 32:3658-3662, 1999; Barrera et al. J. Am. Chem. Soc. 115:11010-11011, 1993; Kwon et al. Macromolecules 22:3250-3255, 1989; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Zhou et al. Macromolecules 23:3399-3406, 1990; each of which is incorporated herein by reference). Examples of these polyesters include poly(L-lactideco-L-lysine) (Barrera et al. J. Am. Chem. Soc. 115:11010-11011, 1993; incorporated herein by reference), poly(serine ester) (Zhou et al. Macromolecules 23:3399-3406, 1990; each of which is incorporated herein by reference), poly(4-hydroxy-L-proline ester) (Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; each of which is incorporated herein by reference), and more recently, poly[α-(4-aminobutyl)-L-glycolic acid]. Poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] were recently demonstrated to condense plasmid DNA through electrostatic interactions, and to mediate gene transfer (Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; each of which is incorporated herein by reference). Importantly, these new polymers are significantly less toxic than poly(lysine) and PEI, and they degrade into non-toxic metabolites. It is clear from these investigations that the rational design of amine-containing polyesters can be a productive route to the development of safe, effective transfection vectors. Unfortunately, however, present syntheses of these polymers require either the independent preparation of specialized monomers (Barrera et al. J. Am. Chem. Soc. 115:11010-11011, 1993; incorporated herein by reference), or the use of stoichiometric amounts of expensive coupling reagents (Putnam et al. Macromolecules 32:3658-3662, 1999; incorporated herein by reference). Additionally, the amine functionalities in the monomers must be protected prior to polymerization (Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Gonzalez et al. Bioconjugate Chem. 10: 1068-1074, 1999; Barrera et al. J. Am. Chem. Soc. 115: 11010-11011, 1993; Kwon et al. Macromolecules 22:3250-3255, 1989; each of which is incorporated herein by reference), necessitating additional post-polymerization deprotection steps before the polymers can be used as transfection agents.

There exists a continuing need for non-toxic, biodegradable, biocompatible polymers that can be used to transfect nucleic acids and that are easily prepared efficiently and economically. Such polymers would have several uses, including the delivery of nucleic acids in gene therapy as well as in the packaging and/or delivery of diagnostic, therapeutic, and prophylactic agents.

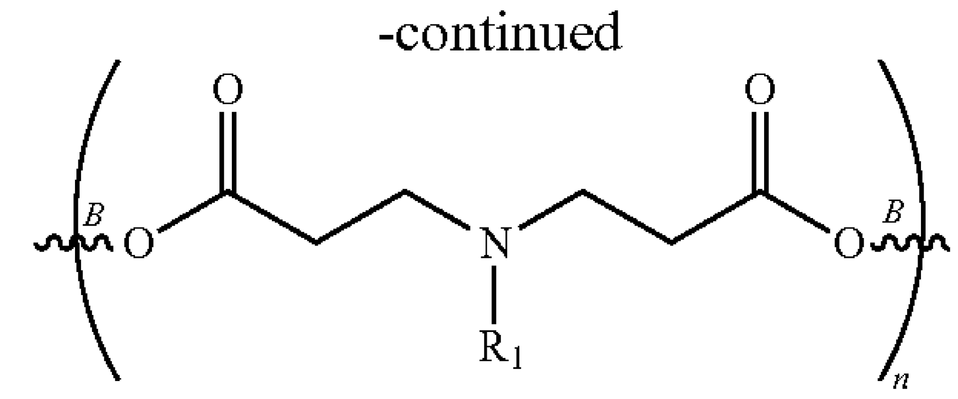
## SUMMARY OF THE INVENTION

The present invention provides polymers useful in preparing drug delivery devices and pharmaceutical compositions thereof. The invention also provides methods of preparing the polymers and methods of preparing microspheres and other pharmaceutical compositions containing the inventive polymers.

The polymers of the present invention are poly(βamino esters) and salts and derivatives thereof. Preferred polymers are biodegradable and biocompatible. Typically, the polymers have one or more tertiary amines in the backbone of the polymer. Preferred polymers have one or two tertiary amines per repeating backbone unit. The polymers may also be copolymers in which one of the components is a poly(β-amino ester). The polymers of the invention may readily be prepared by condensing bis(secondary amines) or primary amines with bis(acrylate esters). A polymer of the invention is represented by either of the formulae below:



-continued



where A and B are linkers which may be any substituted or unsubstituted, branched or unbranched chain of carbon atoms or heteroatoms. The molecular weights of the polymers may range from 1000 g/mol to 20,000 g/mol, preferably from 5000 g/mol to 15,000 g/mol. In certain embodiments, B is an alkyl chain of one to twelve carbons atoms. In other embodiments, B is a heteroaliphatic chain containing a total of one to twelve carbon atoms and heteroatoms. The groups $R_1$ and $R_2$ may be any of a wide variety of substituents. In certain embodiments, $R_1$ and $R_2$ may contain primary amines, secondary amines, tertiary amines, hydroxyl groups, and alkoxy groups. In certain embodiments, the polymers are amine-terminated; and in other embodiments, the polymers are acrylated terminated. In a particularly preferred embodiment, the groups $R_1$ and/or $R_2$ form cyclic structures with the linker A (see the Detailed Description section below). Polymers containing such cyclic moieties have the characteristic of being more soluble at lower pH. Specifically preferred polymers are those that are insoluble in aqueous solutions at physiologic pH (pH 7.2-7.4) and are soluble in aqueous solutions below physiologic pH (pH<7.2). Other preferred polymers are those that are soluble in aqueous solution at physiologic pH (pH 7.2-7.4) and below. Preferred polymers are useful in the transfection of polynucleotides into cells.

In another aspect, the present invention provides a method of preparing the inventive polymers. In a preferred embodiment, commercially available or readily available monomers, bis(secondary amines), primary amines, and bis(acrylate esters), are subjected to conditions which lead to the conjugate addition of the amine to the bis(acrylate ester). In a particularly preferred embodiment, each of the monomers is dissolved in an organic solvent (e.g., DMSO, DMF, THF, diethyl ether, methylene chloride, hexanes, etc.), and the resulting solutions are combined and heated for a time sufficient to lead to polymerization of the monomers. In other embodiments, the polymerization is carried out in the absence of solvent. The resulting polymer may then be purified and optionally characterized using techniques known in the art.

In yet another aspect of the invention, the polymers are used to form nanometer-scale complexes with nucleic acids. The polynucleotide/polymer complexes may be formed by adding a solution of polynucleotide to a vortexing solution of the polymer at a desired DNA/polymer concentration. The weight to weight ratio of polynucleotide to polymer may range from 1:0.1 to 1:200, preferably from 1:10 to 1:150, more preferably from 1:50 to 1:150. The amine monomer to polynucleotide phosphate ratio may be approximately 10: 1, 15:1, 20:1, 25:1, 30:1, 35:1, 40: 1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110:1, 120:1, 130:1, 140:1, 150:1, 160:1, 170:1, 180:1, 190:1, and 200:1. The cationic polymers condense the polynucleotide into soluble particles typically 50-500 nm in size. These polynucleotide/polymer complexes may be used in the delivery of polynucleotides to cells. In a particularly preferred embodiment, these complexes are combined with pharmaceutical excipients to form pharmaceutical compositions suitable for delivery to animals including humans. In certain embodiments, a polymer with a high molecular weight to nitrogen atom ratio (e.g., polylysine, polyethyleneimine) is used to increase transfection efficiency.

In another aspect of the invention, the polymers are used to encapsulate therapeutic, diagnostic, and/or prophylactic agents including polynucleotides to form microparticles. Typically these microparticles are an order of magnitude larger than the polynucleotide/polymer complexes. The microparticles range from 1 micrometer to 500 micrometers. In a particularly preferred embodiment, these microparticles allow for the delivery of labile small molecules, proteins, peptides, and/or polynucleotides to an individual. The microparticles may be prepared using any of the techniques known in the art to make microparticles, such as, for example, double emulsion, phase inversion, and spray drying. In a particularly preferred embodiment, the microparticles can be used for pH-triggered delivery of the encapsulated contents due to the pH-responsive nature of the polymers (i.e., being more soluble at lower pH).

In yet another aspect, the invention provides a system for synthesizing and screening a collection of polymers. In certain embodiments, the system takes advantage of techniques known in the art of automated liquid handling and robotics. The system of synthesizing and screening may be used with poly(beta-amino ester)s as well as other types of polymers including polyamides, polyesters, polyethers, polycarbamates, polycarbonates, polyureas, polyamines, etc. The collection of polymers may be a collection of all one type of polymer (e.g., all poly(betamino esters) or may be a diverse collection of polymers. Thousands of polymers may be synthesized and screened in parallel using the inventive system. In certain embodiments, the polymers are screened for properties useful in the field of gene delivery, transfection, or gene therapy. Some of these properties include solubility at various pHs, ability to complex polynucleotides, ability to transfect a polynucleotide into a cell, etc. Certain poly(beta-amino ester)s found to be useful in transfecting cells include M17, KK89, C32, C36, JJ28, JJ32, JJ36, LL6, and D94 as described in Examples 4 and 5.

## Definitions

The following are chemical terms used in the specification and claims:

The term acyl as used herein refers to a group having the general formula —C(═O)R, where R is alkyl, alkenyl, alkynyl, aryl, carbocylic, heterocyclic, or aromatic heterocyclic. An example of an acyl group is acetyl.

The term alkyl as used herein refers to saturated, straight- or branched-chain hydrocarbon radicals derived from a hydrocarbon moiety containing between one and twenty carbon atoms by removal of a single hydrogen atom. In some embodiments, the alkyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkyl group employed contains 1-8 carbon atoms. In still other embodiments, the alkyl group contains 1-6 carbon atoms. In yet another embodiments, the alkyl group contains 1-4 carbons. Examples of alkyl radicals include, but are not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, iso-butyl, sec-butyl, sec-pentyl, iso-pentyl, tert-butyl, n-pentyl, neopentyl, n-hexyl, sec-hexyl, n-heptyl, n-octyl, n-decyl, n-undecyl, dodecyl, and the like, which may bear one or more sustitutents.

The term alkoxy as used herein refers to a saturated (i.e., alkyl-O—) or unsaturated (i.e., alkenyl-O— and alkynyl-O—) group attached to the parent molecular moiety through an oxygen atom. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the akyl, akenyl, and alkynyl groups employed in the invention contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contains 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl group contains 1-4 aliphatic carbon atoms. Examples include, but are not limited to, methoxy, ethoxy, propoxy, isopropoxy, n-butoxy, tert-butoxy, i-butoxy, sec-butoxy, neopentoxy, n-hexoxy, and the like.

The term alkenyl denotes a monovalent group derived from a hydrocarbon moiety having at least one carbon-carbon double bond by the removal of a single hydrogen atom. In certain embodiments, the alkenyl group employed in the invention contains 1-20 carbon atoms. In some embodiments, the alkenyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkenyl group employed contains 1-8 carbon atoms. In still other embodiments, the alkenyl group contains 1-6 carbon atoms. In yet another embodiments, the alkenyl group contains 1-4 carbons. Alkenyl groups include, for example, ethenyl, propenyl, butenyl, 1-methyl-2-buten-1-yl, and the like.

The term alkynyl as used herein refers to a monovalent group derived form a hydrocarbon having at least one carbon-carbon triple bond by the removal of a single hydrogen atom. In certain embodiments, the alkynyl group employed in the invention contains 1-20 carbon atoms. In some embodiments, the alkynyl group employed in the invention contains 1-10 carbon atoms. In another embodiment, the alkynyl group employed contains 1-8 carbon atoms. In still other embodiments, the alkynyl group contains 1-6 carbon atoms. Representative alkynyl groups include, but are not limited to, ethynyl, 2-propynyl (propargyl), 1-propynyl, and the like.

The term alkylamino, dialkylamino, and trialkylamino as used herein refers to one, two, or three, respectively, alkyl groups, as previously defined, attached to the parent molecular moiety through a nitrogen atom. The term alkylamino refers to a group having the structure—NHR' wherein R' is an alkyl group, as previously defined; and the term dialkylamino refers to a group having the structure —NR'R", wherein R' and R' are each independently selected from the group consisting of alkyl groups. The term trialkylamino refers to a group having the structure —NR'R"R'", wherein R', R", and R'" are each independently selected from the group consisting of alkyl groups. In certain embodiments, the alkyl group contain 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl group contains 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl group contain 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl group contain 1-4 aliphatic carbon atoms. Additionally, R', R", and/or R'" taken together may optionally be —$(CH_2)_k$— where k is an integer from 2 to 6. Examples include, but are not limited to, methylamino, dimethylamino, ethylamino, diethylamino, diethylaminocarbonyl, methylethylamino, iso-propylamino, piperidino, trimethylamino, and propylamino.

The terms alkylthioether and thioalkoxyl refer to a saturated (i.e., alkyl-S—) or unsaturated (i.e., alkenyl-S— and alkynyl-S—) group attached to the parent molecular moiety through a sulfur atom. In certain embodiments, the alkyl group contains 1-20 aliphatic carbon atoms. In certain other embodiments, the alkyl group contains 1-10 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups contain 1-8 aliphatic carbon atoms. In still other embodiments, the alkyl, alkenyl, and alkynyl groups contain 1-6 aliphatic carbon atoms. In yet other embodiments, the alkyl, alkenyl, and alkynyl groups contain 1-4 aliphatic carbon atoms. Examples of thioalkoxyl moieties include, but are not limited to, methylthio, ethylthio, propylthio, isopropylthio, n-butylthio, and the like.

The term aryl as used herein refers to an unsaturated cyclic moiety comprising at least one aromatic ring. In certain embodiments, aryl group refers to a mono- or bicyclic carbocyclic ring system having one or two aromatic rings including, but not limited to, phenyl, naphthyl, tetrahydronaphthyl, indanyl, indenyl, and the like. Aryl groups can be unsubstituted or substituted with substituents selected from the group consisting of branched and unbranched alkyl, alkenyl, alkynyl, haloalkyl, alkoxy, thioalkoxy, amino, alkylamino, dialkylamino, trialkylamino, acylamino, cyano, hydroxy, halo, mercapto, nitro, carboxyaldehyde, carboxy, alkoxycarbonyl, and carboxamide. In addition, substituted aryl groups include tetrafluorophenyl and pentafluorophenyl.

The term carboxylic acid as used herein refers to a group of formula —$CO_2H$.

The terms halo and halogen as used herein refer to an atom selected from fluorine, chlorine, bromine, and iodine.

The term heterocyclic, as used herein, refers to an aromatic or non-aromatic, partially unsaturated or fully saturated, 3- to 10-membered ring system, which includes single rings of 3 to 8 atoms in size and bi- and tri-cyclic ring systems which may include aromatic five- or six-membered aryl or aromatic heterocyclic groups fused to a non-aromatic ring. These heterocyclic rings include those having from one to three heteroatoms independently selected from oxygen, sulfur, and nitrogen, in which the nitrogen and sulfur heteroatoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. In certain embodiments, the term heterocylic refers to a non-aromatic 5-, 6-, or 7-membered ring or a polycyclic group wherein at least one ring atom is a heteroatom selected from O, S, and N (wherein the nitrogen and sulfur heteroatoms may be optionally oxidized), including, but not limited to, a bi- or tri-cyclic group, comprising fused six-membered rings having between one and three heteroatoms independently selected from the oxygen, sulfur, and nitrogen, wherein (i) each 5-membered ring has 0 to 2 double bonds, each 6-membered ring has 0 to 2 double bonds, and each 7-membered ring has 0 to 3 double bonds, (ii) the nitrogen and sulfur heteroatoms may be optionally oxidized, (iii) the nitrogen heteroatom may optionally be quaternized, and (iv) any of the above heterocyclic rings may be fused to an aryl or heteroaryl ring.

The term aromatic heterocyclic, as used herein, refers to a cyclic aromatic radical having from five to ten ring atoms of which one ring atom is selected from sulfur, oxygen, and nitrogen; zero, one, or two ring atoms are additional heteroatoms independently selected from sulfur, oxygen, and nitrogen; and the remaining ring atoms are carbon, the radical being joined to the rest of the molecule via any of the ring atoms, such as, for example, pyridyl, pyrazinyl, pyrimidinyl, pyrrolyl, pyrazolyl, imidazolyl, thiazolyl, oxazolyl, isooxazolyl, thiadiazolyl, oxadiazolyl, thiophenyl, furanyl, quinolinyl, isoquinolinyl, and the like. Aromatic heterocyclic groups can be unsubstituted or substituted with substituents selected from the group consisting of branched and unbranched alkyl, alkenyl, alkynyl, haloalkyl, alkoxy, thioalkoxy, amino, alkylamino, dialkylamino, trialkylamino, acylamino, cyano, hydroxy, halo, mercapto, nitro, carboxyaldehyde, carboxy, alkoxycarbonyl, and carboxamide.

Specific heterocyclic and aromatic heterocyclic groups that may be included in the compounds of the invention include: 3-methyl-4-(3-methylphenyl)piperazine, 3 methylpiperidine, 4-(bis-(4-fluorophenyl)methyl)piperazine, 4-(diphenylmethyl)piperazine, 4(ethoxycarbonyl)piperazine, 4-(ethoxycarbonylmethyl)piperazine, 4-(phenylmethyl)piperazine, 4-(1-phenylethyl)piperazine, 4-(1,1-dimethylethoxycarbonyl)piperazine, 4-(2-(bis-(2-propenyl) amino)ethyl)piperazine, 4-(2-(diethylamino)ethyl)piperazine, 4-(2-chlorophenyl)piperazine, 4(2-cyanophenyl)piperazine, 4-(2-ethoxyphenyl)piperazine, 4-(2-ethylphenyl) piperazine, 4-(2-fluorophenyl)piperazine, 4-(2-hydroxyethyl)piperazine, 4-(2-methoxyethyl)piperazine, 4-(2-methoxyphenyl)piperazine, 4-(2-methylphenyl)piperazine, 4-(2-methylthiophenyl) piperazine, 4(2-nitrophenyl) piperazine, 4-(2-nitrophenyl)piperazine, 4-(2-phenylethyl) piperazine, 4-(2-pyridyl)piperazine, 4-(2-pyrimidinyl) piperazine, 4-(2,3-dimethylphenyl)piperazine, 4-(2,4-difluorophenyl) piperazine, 4-(2,4-dimethoxyphenyl) piperazine, 4-(2,4-dimethylphenyl)piperazine, 4-(2,5-dimethylphenyl)piperazine, 4-(2,6-dimethylphenyl) piperazine, 4-(3-chlorophenyl)piperazine, 4-(3-methylphenyl)piperazine, 4-(3-trifluoromethylphenyl) piperazine, 4-(3,4-dichlorophenyl)piperazine, 4-3,4-dimethoxyphenyl)piperazine, 4-(3,4-dimethylphenyl) piperazine, 4-(3,4-methylenedioxyphenyl)piperazine, 4-(3,4,5-trimethoxyphenyl)piperazine, 4-(3,5-dichlorophenyl) piperazine, 4-(3,5-dimethoxyphenyl)piperazine, 4-(4-(phenylmethoxy)phenyl)piperazine, 4-(4-(3,1-dimethylethyl)phenylmethyl)piperazine, 4-(4-chloro-3-trifluoromethylphenyl)piperazine, 4-(4-chlorophenyl)-3-methylpiperazine, 4-(4-chlorophenyl)piperazine, 4-(4-chlorophenyl)piperazine, 4-(4-chlorophenylmethyl) piperazine, 4-(4-fluorophenyl)piperazine, 4-(4-methoxyphenyl)piperazine, 4-(4-methylphenyl)piperazine, 4-(4-nitrophenyl)piperazine, 4-(4-trifluoromethylphenyl) piperazine, 4-cyclohexylpiperazine, 4-ethylpiperazine, 4-hydroxy-4-(4-chlorophenyl)methylpiperidine, 4-hydroxy-4-phenylpiperidine, 4-hydroxypyrrolidine, 4-methylpiperazine, 4-phenylpiperazine, 4-piperidinylpiperazine, 4-(2-furanyl)carbonyl)piperazine, 4-((1,3-dioxolan-5-yl)methyl)piperazine, 6-fluoro-1,2,3,4-tetrahydro-2-methylquinoline, 1,4-diazacylcloheptane, 2,3-dihydroindolyl, 3,3-dimethylpiperidine, 4,4-ethylenedioxypiperidine, 1,2,3,4-tetrahydroisoquinoline, 1,2,3,4-tetrahydroquinoline, azacyclooctane, decahydroquinoline, piperazine, piperidine, pyrrolidine, thiomorpholine, and triazole.

The term carbamoyl, as used herein, refers to an amide group of the formula —$CONH_2$.

The term carbonyldioxyl, as used herein, refers to a carbonate group of the formula —O—CO—OR.

The term hydrocarbon, as used herein, refers to any chemical group comprising hydrogen and carbon. The hydrocarbon may be substituted or unsubstitued. The hydrocarbon may be unsaturated, saturated, branched, unbranched, cyclic, polycyclic, or heterocyclic. Illustrative hydrocarbons include, for example, methyl, ethyl, n-propyl, iso-propyl, cyclopropyl, allyl, vinyl, n-butyl, tert-butyl, ethynyl, cyclohexyl, methoxy, diethylamino, and the like. As would be known to one skilled in this art, all valencies must be satisfied in making any substitutions.

The terms substituted, whether preceded by the term "optionally" or not, and substituent, as used herein, refer to the ability, as appreciated by one skilled in this art, to change one functional group for another functional group provided that the valency of all atoms is maintained. When more than one position in any given structure may be substituted with more than one substituent selected from a specified group, the substituent may be either the same or different at every position. The substituents may also be further substituted (e.g., an aryl group substituent may have another substituent off it, such as another aryl group, which is further substituted with fluorine at one or more positions).

The term thiohydroxyl or thiol, as used herein, refers to a group of the formula —SH.

The term ureido, as used herein, refers to a urea group of the formula —NH—CO—$NH_2$.

The following are more general terms used throughout the present application:

"Animal": The term animal, as used herein, refers to humans as well as non-human animals, including, for example, mammals, birds, reptiles, amphibians, and fish. Preferably, the non-human animal is a mammal (e.g., a rodent, a mouse, a rat, a rabbit, a monkey, a dog, a cat, a primate, or a pig). An animal may be a domesticated animal. An animal may be a transgenic animal.

"Associated with": When two entities are "associated with" one another as described herein, they are linked by a direct or indirect covalent or non-covalent interaction. Preferably, the association is covalent. Desirable non-covalent interactions include hydrogen bonding, van der Waals interactions, hydrophobic interactions, magnetic interactions, electrostatic interactions, etc.

"Biocompatible": The term "biocompatible", as used herein is intended to describe compounds that are not toxic to cells. Compounds are "biocompatible" if their addition to cells in vitro results in less than or equal to 20% cell death, and their administration in vivo does not induce inflammation or other such adverse effects.

"Biodegradable": As used herein, "biodegradable" compounds are those that, when introduced into cells, are broken down by the cellular machinery or by hydrolysis into components that the cells can either reuse or dispose of without significant toxic effect on the cells (i.e., fewer than about 20% of the cells are killed when the components are added to cells in vitro). The components preferably do not induce inflammation or other adverse effects in vivo. In certain preferred embodiments, the chemical reactions relied upon to break down the biodegradable compounds are uncatalyzed.

"Effective amount": In general, the "effective amount" of an active agent or drug delivery device refers to the amount necessary to elicit the desired biological response. As will be appreciated by those of ordinary skill in this art, the effective amount of an agent or device may vary depending on such factors as the desired biological endpoint, the agent to be delivered, the composition of the encapsulating matrix, the target tissue, etc. For example, the effective amount of microparticles containing an antigen to be delivered to immunize an individual is the amount that results in an immune response sufficient to prevent infection with an organism having the administered antigen.

"Peptide" or "protein": According to the present invention, a "peptide" or "protein" comprises a string of at least three amino acids linked together by peptide bonds. The terms "protein" and "peptide" may be used interchangeably. Peptide may refer to an individual peptide or a collection of peptides. Inventive peptides preferably contain only natural amino acids, although non-natural amino acids (i.e., compounds that do not occur in nature but that can be incorporated into a polypeptide chain) and/or amino acid analogs as are known in the art may alternatively be employed. Also, one or more of the amino acids in an inventive peptide may be modified, for example, by the addition of a chemical entity such as a carbohydrate group, a phosphate group, a farnesyl group, an isofarnesyl group, a fatty acid group, a linker for conjugation, functionalization, or other modification, etc. In a preferred embodiment, the modifications of the peptide lead to a more stable peptide (e.g., greater half-life in vivo). These modifications may include cyclization of the peptide, the incorporation of D-amino acids, etc. None of the modifications should substantially interfere with the desired biological activity of the peptide.

"Polynucleotide" or "oligonucleotide": Polynucleotide or oligonucleotide refers to a polymer of nucleotides. Typically, a polynucleotide comprises at least three nucleotides. The polymer may include natural nucleosides (i.e., adenosine, thymidine, guanosine, cytidine, uridine, deoxyadenosine, deoxythymidine, deoxyguanosine, and deoxycytidine), nucleoside analogs (e.g., 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, C5-propynylcytidine, C5-propynyluridine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, 0(6)-methylguanine, and 2-thiocytidine), chemically modified bases, biologically modified bases (e.g., methylated bases), intercalated bases, modified sugars (e.g., 2'-fluororibose, ribose, 2'-deoxyribose, arabinose, and hexose), or modified phosphate groups (e.g., phosphorothioates and 5'-N-phosphoramidite linkages).

"Small molecule": As used herein, the term "small molecule" refers to organic compounds, whether naturally-occurring or artificially created (e.g., via chemical synthesis) that have relatively low molecular weight and that are not proteins, polypeptides, or nucleic acids. Typically, small molecules have a molecular weight of less than about 1500 g/mol. Also, small molecules typically have multiple carbon-carbon bonds. Known naturally-occurring small molecules include, but are not limited to, penicillin, erythromycin, taxol, cyclosporin, and rapamycin. Known synthetic small molecules include, but are not limited to, ampicillin, methicillin, sulfamethoxazole, and sulfonamides.

## BRIEF DESCRIPTION OF THE DRAWING

FIG. **1** shows the time profile for the degradation of polymers 1-3 at 37° C. at pH 5.1 and pH 7.4. Degradation is expressed as percent degradation over time based on GPC data.

FIG. **2** shows cytotoxicity profiles of polymers 1-3 and PEI. Viability of NIH 3T3 cells is expressed as a function of polymer concentration. The molecular weights of polymers 1, 2, and 3 were 5800, 11300, and 22500, respectively. The molecular weight of the PEI employed was 25000.

FIG. **3** shows the retardation of pCMV-Luc DNA by polymer 1 in agarose gel electrophoresis. Each lane corresponds to a different DNA/polymer weight ratio. The ratios are as follows: 1) 1:0 (DNA only); 2) 1:0.5; 3) 1:1; 4) 1:2; 5) 1:3; 6) 1:4; 7) 1:5; 8) 1:6; 9) 1:7; and 10) 1:8.

FIG. **4** shows the average effective diameters of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer 3 ($M_n$=31,000) as a function of polymer concentration.

FIG. **5** shows average ζ-potentials of DNA/polymer complexes formed from pCMV-Luc plasmid and polymer 3 ($M_n$=31,000) as a function of polymer concentration. The numbers for each complex correspond to the complex numbers in FIG. **4**.

FIG. **6** is an SEM image of rhodamine/dextran-loaded microspheres fabricated from polymer 1.

FIG. **7** shows the release profiles of rhodamine/dextran from polymer 1 and PLGA microspheres at various pH values. The arrows indicate the points at which HEPES buffer (pH 7.4) was exchanged with acetate buffer (pH 5.1).

FIG. **8** shows a) a representative fluorescence microscopy image of rhodamine/dextran-loaded polymer 1 microspheres suspended in HEPES buffer (pH 7.4). FIG. **8**b shows a sample of loaded polymer 1 microspheres at pH 7.4 after addition of acetate buffer (pH 5.1). The direction of diffusion of acid is from the top right to the bottom left of the image (elapsed time seconds).

FIG. **9** demonstrates the gel electrophoresis assay used to identify DNA-complexing polymers. Lane annotations correspond to the 70 water-soluble members of the screening library. For each polymer, assays were performed at DNA/polymer ratios of 1:5 (left well) and 1:20 (right well). Lanes marked C* contain DNA alone (no polymer) and were used as a control.

FIG. **10** shows transfection data as a function of structure for an assay employing pCMV-Luc (600 ng/well, DNA/polymer=1:20). Light units are arbitrary and not normalized to total cell protein; experiments were performed in triplicate (error bars not shown). Black squares represent water-insoluble polymers, white squares represent water-soluble polymers that did not complex DNA in FIG. **9**. The right column (marked "*") displays values for the following control experiments: no polymer (green), PEI (red), and Lipofectamine (light blue).

FIG. **11** shows a synthesis of poly(beta-amino ester)s. Poly (beta-amino ester)s may be synthesized by the conjugate addition of primary amines (equation 1) or bis(secondary amines) (equation 2) to diacrylates.

FIG. **12** shows a variety of amine (A) and diacrylate (B) monomers used in the synthesis of the polymer library.

FIG. **13** is a histogram of polymer transfection efficiencies. In the first screen all 2350 polymers were tested for their ability to deliver pCMV-luc DNA at N:P ratios of 40:1, 20: 1, and 10:1 to COS-7 cells. Transfection efficiency is presented in ng Luciferase per well. For reference, PEI transfection efficiency is shown. COS-7 cells readily take up naked DNA, and in our conditions produce 0.15±0.05 ng per well, and the lipid reagent, Lipofectamine 2000, produces 13.5±1.9 ng per well.

FIG. **14**. A) Optimized transfection efficiency of the top 50 polymers relative to PEI and lipofectamine 2000. Polymers were tested as described in methods. In the first broad screen N:P ratios of 40:1, 20: 1, and 10:1 with an n of 1 were tested. The top 93 were rescreened at six different N:P ratios=(optimal N:P form the first screen)×1.75, 1.5, 1.25, 1.0, 0.75, and 0.5, in triplicate. Control reactions are labeled in Red, and polymers that did not bind DNA in a gel electrophoresis assay are shown in black. B) DNA binding polymers as determined by agarose gel electrophoresis. The data was tabulated in the following manner: 1) fully shifted DNA is represented by (+), 2) partially shifted DNA is represented by (+/−), 3) unbound DNA is represented by (−).

FIG. **15** shows the transfection of COS-7 cells using enhanced Green Fluorescent Protein plasmid. Cells were transfected at an N:P ratio of (optimal N:P from the broad screen)×1.25 with 600 ng of DNA. Regions of the well showing high transfection are shown for the following polymers: a) C36, b) D94.

FIG. **16** shows how the polymer molecular weight and the chain end-group is affected by varying the amine/diacrylate ratio in the reaction mixture. Molecular weights (Mw) (relative to polystyrene standards) were determined by organic phase GPC. Polymers synthesized with amine/diacrylate ratios of >1 have amine end-groups, and polymers synthesized with amine/diacrylate ratios of <1 have acrylate end-groups.

FIG. **17** shows luciferase transfection results for Poly-1 as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

FIG. **18** shows luciferase transfection results for Poly-2 as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n—4, error bars not shown).

FIG. **19** shows the cytotoxicity of poly-1/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

FIG. **20** shows the cytotoxicity of poly-2/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

FIG. **21** shows the relative cellular uptake level of poly-1/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains; (B) acrylate-terminated chains. (n=4, error bars are not shown.)

FIG. **22** shows the relative cellular uptake level of poly-2/DNA complexes as a function of polymer molecular weight, polymer/DNA ratio (w/w), and polymer end-group. (A) amine-terminated chains (blank squares represent conditions where cytotoxicity of the complexes prevented a reliable measurement of cellular uptake); (B) acrylate-terminated chains. (n=4, error bars not shown.)

FIG. **23** shows the enhancement of transfection activity of poly-1 (amine-terminated chains, $M_W$=13,100) based delivery vectors through the use of co-complexing agents. (A) polylysine (PLL); (B) polyethyleneimine (PEI). (n=4, error bars are not shown).

FIG. **24** shows the enhancement of transfection activity of poly-2 (amine-terminated chains, MW=13,400) based delivery vectors through the use of co-complexing agents. (A) polylysine (PLL); (B) polyethyleneimine (PEI). (n=4, error bars are not shown.)

FIG. **25** is a comparison of GFP gene transfer into COS-7 cells using Poly-1/PLL (Poly-1:PLL:DNA=60:0.1:1 (w/w/w)), Poly-2/PLL (Poly-2:PLL:DNA=15:0.4:1 (w/w/w)), Lipofectamine 2000 (μL reagent: μg DNA=1:1), PEI (PEI:DNA 1:1 (w/w), N/P 8), and naked DNA. Cells were seeded on 6-well plates and grown to new confluence. Cells were the incubated with complexes (5 μg DNA/well) for 1 hour, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n=3, error bars indicate one standard deviation.)

FIG. **26** shows GFP expression in COS-7 cells transfected using Poly-1/PLL.

FIG. **27** shows GFP gene transfer into four different cell lines using Poly-1/PLL (Poly-1:PLL:DNA=60:0.1:1 (w/w/w). Cells were seeded on 6-well plates and grown to near confluence. Cells were then incubated with complexes (5 μg DNA/well) for 1 hour, after which time complexes were removed and fresh growth media was added. Two days later GFP expression was assayed by flow cytometry. (n=5, error bars indicate one standard deviation.)

## DETAILED DESCRIPTION OF CERTAIN PREFERRED EMBODIMENTS OF THE INVENTION

The present invention provides improved polymeric encapsulation and delivery systems based on the use of β-amino ester polymers. The sytems may be used in the pharmaceutical/drug delivery arts to delivery polynucleotides, proteins, small molecules, peptides, antigen, drugs, etc. to a patient, tissue, organ, cell, etc. The present invention also provides for the preparation and screening of large collections of polymers for "hits" that are useful in the pharmaceutical and drug delivery arts.

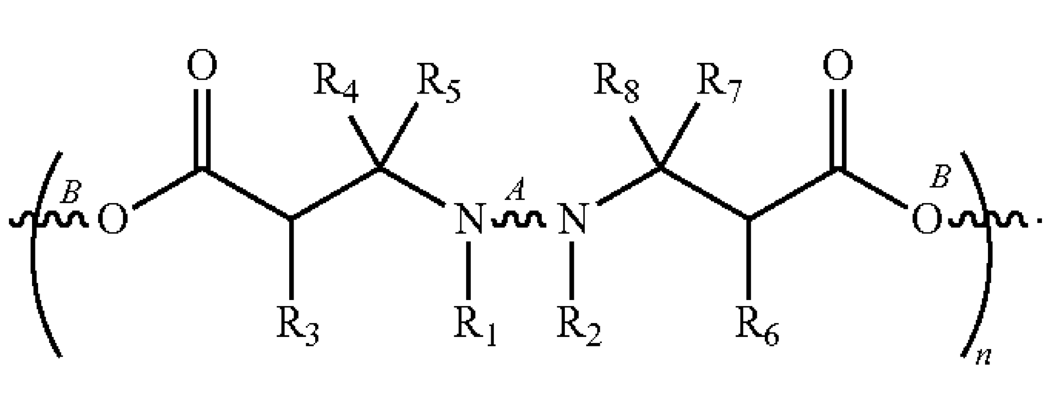
The β-amino ester polymers of the present invention provide for several different uses in the drug delivery art. The polymers with their tertiary amine-containing backbones may be used to complex polynucleotides and thereby enhance the delivery of polynucleotide and prevent their degradation. The polymers may also be used in the formation of nanoparticles or microparticles containing encapsulated agents. Due to the polymers' properties of being biocompatible and biodegradable, these formed particles are also biodegradable and biocompatible and may be used to provide controlled, sustained release of the encapsulated agent. These particles may also be responsive to pH changes given the fact that these polymers are typically not substantially soluble in aqueous solution at physiologic pH but are more soluble at lower pH.

Polymers

The polymers of the present invention are poly(β-amino esters) containing tertiary amines in their backbones and salts thereof. The molecular weights of the inventive polymers may range from 5,000 g/mol to over 100,000 g/mol, more preferably from 4,000 g/mol to 50,000 g/mol. In a particularly preferred embodiment, the inventive polymers are relatively non-cytotoxic. In another particularly preferred embodiment, the inventive polymers are biocompatible and biodegradable. In a particularly preferred embodiment, the polymers of the present invention have $pK_a$s in the range of 5.5 to 7.5, more preferably between 6.0 and 7.0. In another particularly preferred embodiment, the polymer may be designed to have a desired $pK_a$ between 3.0 and 9.0, more preferably between 5.0 and 8.0. The inventive polymers are particularly attractive for drug delivery for several reasons: 1) they contain amino groups for interacting with DNA and other negatively charged agents, for buffering the pH, for causing endosomolysis, etc.; 2) they contain degradable polyester linkages; 3) they can be synthesized from commercially available starting materials; and 4) they are pH responsive and future generations could be engineered with a desired $pK_a$. In screening for transfection efficiency, the best performing polymers were hydrophobic or the diacrylate monomers were hydrophobic, and many had mono- or di-hydroxyl side chains and/or linear, bis(secondary amines) as part of their structure.

The polymers of the present invention can generally be defined by the formula (I):

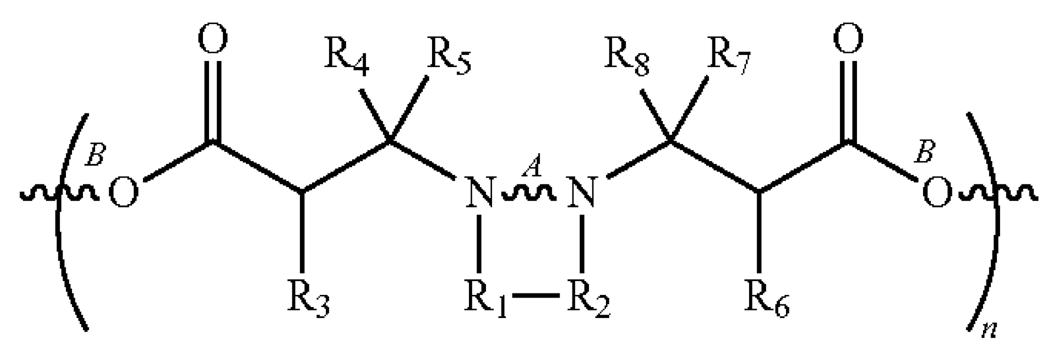
(I)



The linkers A and B are each a chain of atoms covalently linking the amino groups and ester groups, respectively. These linkers may contain carbon atoms or heteroatoms (e.g., nitrogen, oxygen, sulfur, etc.). Typically, these linkers are 1 to 30 atoms long, more preferably 1-15 atoms long. The linkers may be substituted with various substituents including, but not limited to, hydrogen atoms, alkyl, alkenyl, alkynl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, thioether, alkylthioether, thiol, and ureido groups. As would be appreciated by one of skill in this art, each of these groups may in turn be substituted. The groups $R_1$, $R_2$, $R_3$, $R_4$, $R_5$, $R_6$, $R_7$, and $R_8$ may be any chemical groups including, but not limited to, hydrogen atoms, alkyl, alkenyl, alkynl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, alkylthioether, thiol, and ureido groups. In the inventive polymers, n is an integer ranging from 5 to 10,000, more preferably from 10 to 500.

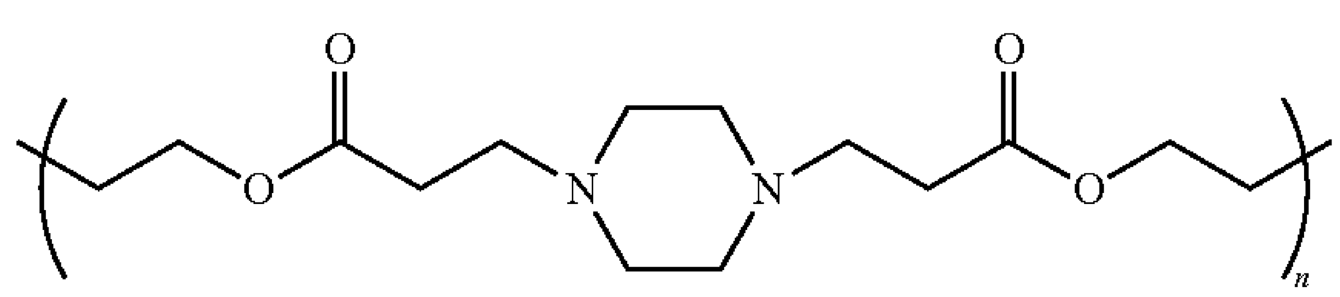
In a particularly preferred embodiment, the bis(secondary amine) is a cyclic structure, and the polymer is generally represented by the formula II:

(II)

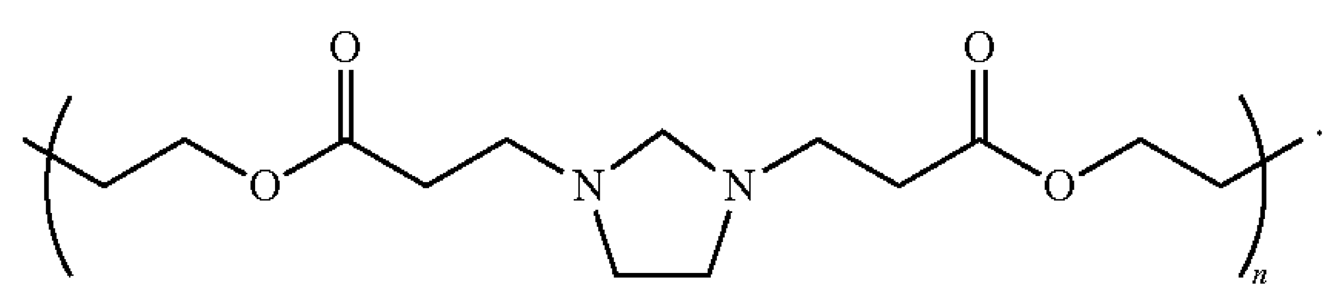


In this embodiment, $R_1$ and $R_2$ are directly linked together as shown in formula II. Examples of inventive polymers in this embodiment include, but are not limited to formulas III and IV:

(III)



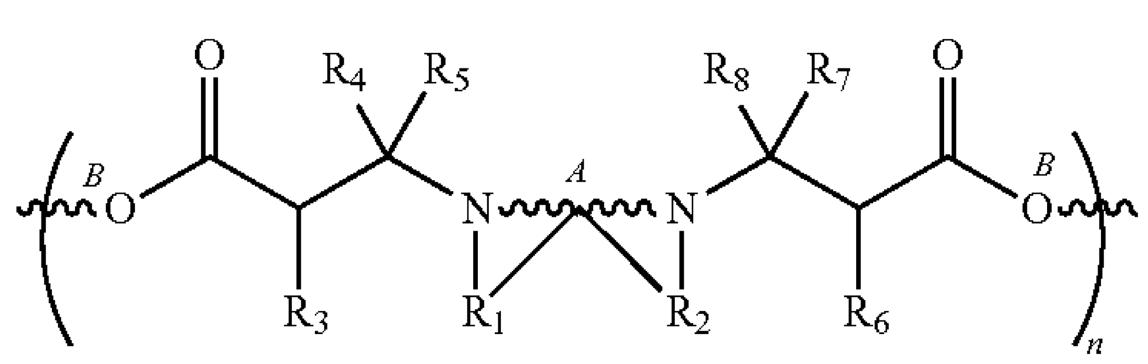
(IV)



As described above in the preceding paragraph, any chemical group that satisfies the valency of each atom may be substituted for any hydrogen atom.

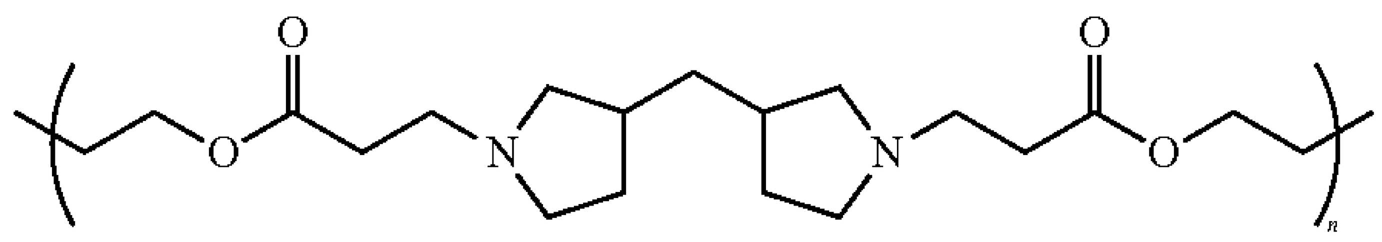
In another particularly preferred embodiment, the groups $R_1$ and/or $R_2$ are covalently bonded to linker A to form one or two cyclic structures. The polymers of the present embodiment are generally represented by the formula V in which both $R_1$ and $R_2$ are bonded to linker A to form two cyclic structures:

(V)

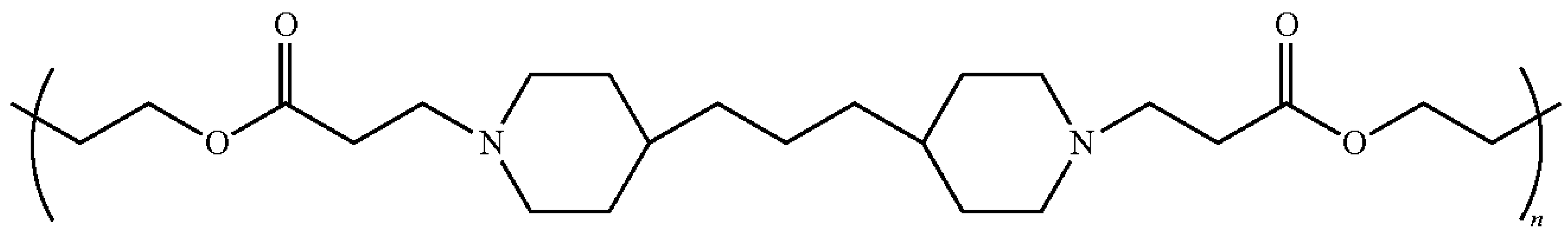


The cyclic structures may be 3-, 4-, 5-, 6-, 7-, or 8-membered rings or larger. The rings may contain heteroatoms and be unsaturated. Examples of polymers of formula V include formulas VI, VII, and VIII:

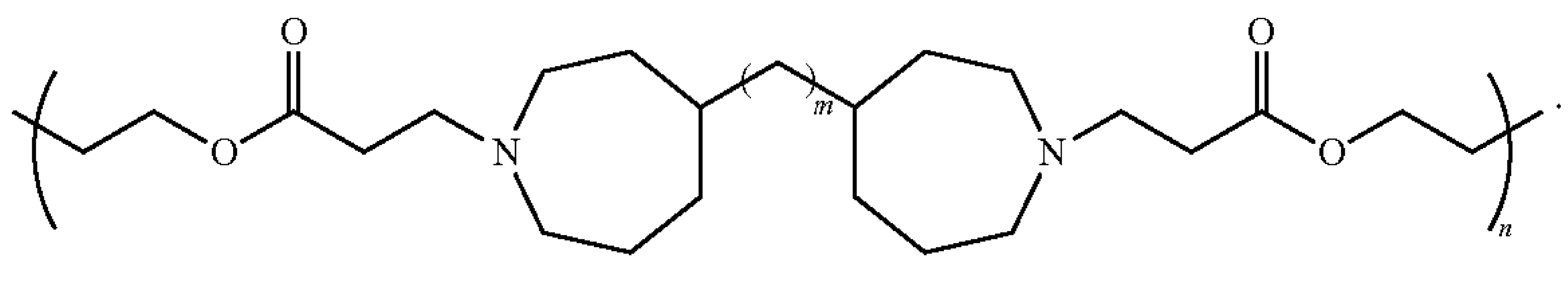
(VI)



(VII)



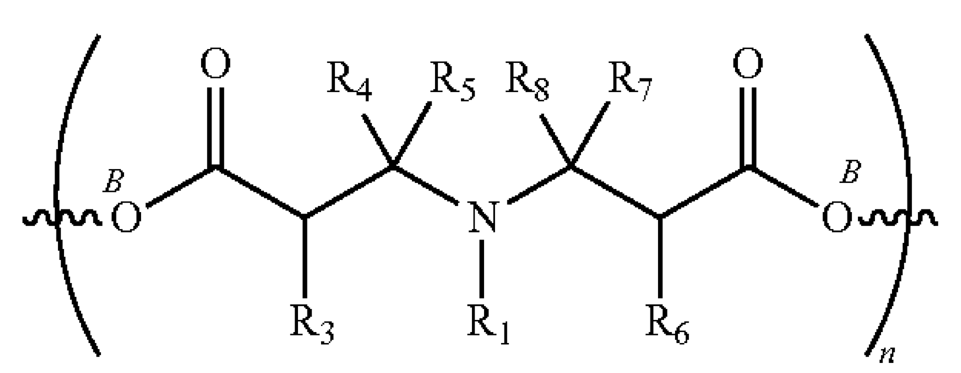
(VIII)



As described above, any chemical group that satisfies the valency of each atom in the molecule may be substituted for any hydrogen atom.

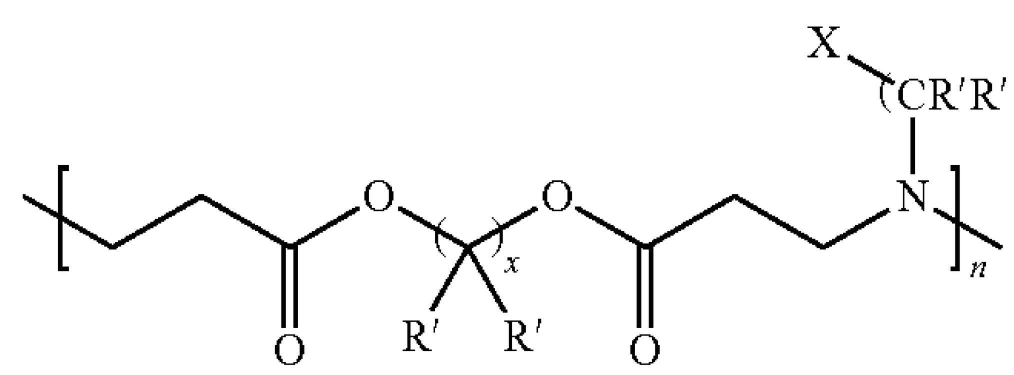
In another embodiment, the polymers of the present invention can generally be defined by the formula (IX):

(IX)



The linker B is a chain of atoms covalently linking the ester groups. The linker may contain carbon atoms or heteroatoms (e.g., nitrogen, oxygen, sulfur, etc.). Typically, the linker is 1 to 30 atoms long, preferably 1-15 atoms long, and more preferably 2-10 atoms long. In certain embodiments, the linker B is a substituted or unsubstituted, linear or branched alkyl chain, preferably with 3-10 carbon atoms, more preferably with 3, 4, 5, 6, or 7 carbon atoms. In other embodiments, the linker B is a substituted or unsubstituted, linear or branched heteroaliphatic chain, preferably with 3-10 atoms, more preferably with 3, 4, 5, 6, or 7 atoms. In certain embodiments, the linker B is comprises of repeating units of oxygen and carbon atoms. The linker may be substituted with various substituents including, but not limited to, hydrogen atoms, alkyl, alkenyl, alkynyl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, thioether, alkylthioether, thiol, acyl, acetyl, and ureido groups. As would be appreciated by one of skill in this art, each of these groups may in turn be substituted. Each of R1, R3, R4, R5, R6, R7, and R8 may be independently any chemical group including, but not limited to, hydrogen atom, alkyl, alkenyl, alkynyl, amino, alkylamino, dialkylamino, trialkylamino, hydroxyl, alkoxy, halogen, aryl, heterocyclic, aromatic heterocyclic, cyano, amide, carbamoyl, carboxylic acid, ester, alkylthioether, thiol, acyl, acetyl, and ureido groups. In certain embodiments, R1 includes hydroxyl groups. In other embodiments, R1 includes amino, alkylamino, or dialkylamino groups. In the inventive polymer, n is an integer ranging from 5 to 10,000, more preferably from 10 to 500.

In certain embodiments, the polymers of the present invention are generally defined as follows:



wherein

X is selected from the group consiting of $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, halogen, OR and $NR_2$; more preferably, methyl, OH, or $NH_2$;

R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;

each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen; preferably, R' is hydrogen, methyl, ethyl, propyl, isopropyl, butyl, methoxy, ethoxy, propoxy, isopropoxy, hydroxyl, amino, fluoro, chloro, or bromo; more preferably, R' is fluoro, hydrogen, or methyl;

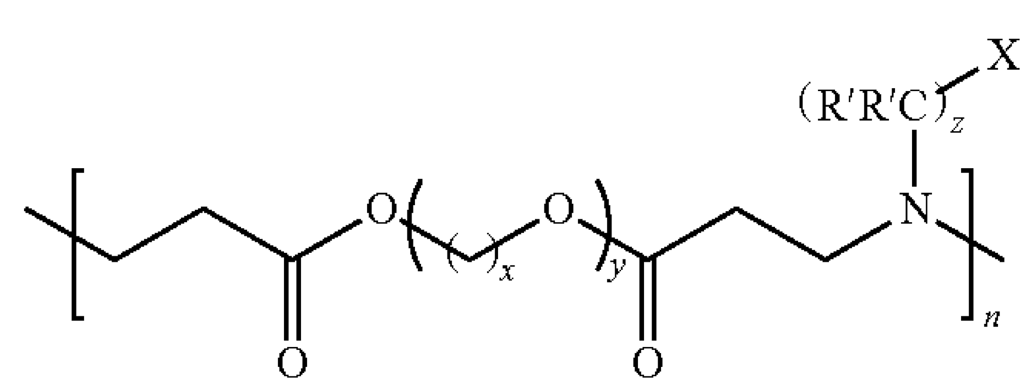
n is an integer between 3 and 10,000;

x is an integer between 1 and 10; preferably, x is an integer between 2 and 6;

y is an integer between 1 and 10; preferably, x is an interger between 2 and 6; and

derivatives and salts thereof.

In certain embodiments, the polymers of the present invention are generally defined as follows:



wherein

X is selected from the group consiting of $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, halogen, OR and $NR_2$; more preferably, methyl, OH, or $NH_2$;

R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;

each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen; preferably, R' is hydrogen, methyl, ethyl, propyl, isopropyl, butyl, methoxy, ethoxy, propoxy, isopropoxy, hydroxyl, amino, fluoro, chloro, or bromo; more preferably, R' is fluoro, hydrogen, or methyl;

n is an integer between 3 and 10,000;

x is an integer between 1 and 10; preferably, x is an integer between 2 and 6;

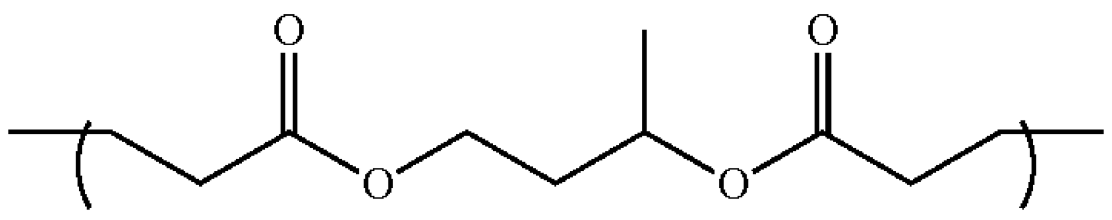
y is an integer between 1 and 10; preferably, y is an interger between 2 and 6;

z is an interger between 1 and 10; preferably, z is an integer between 2 and 6; and

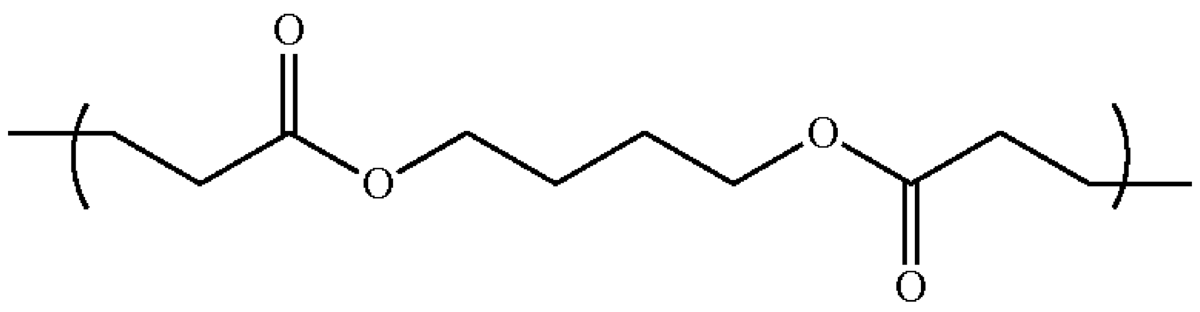
derivatives and salts thereof.

In another embodiment, the bis(acrylate ester) unit in the inventive polymer is chosen from the following group of bis(acrylate ester) units (A'-G'):

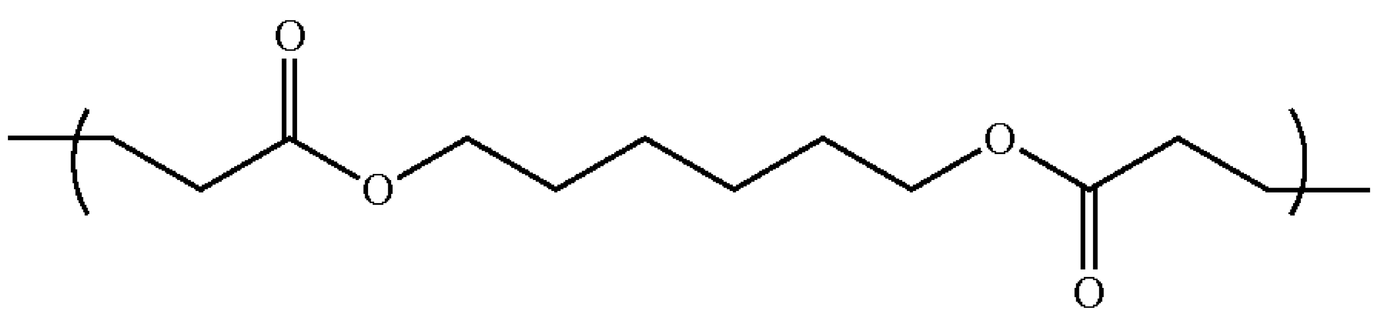
A'



B'



C'





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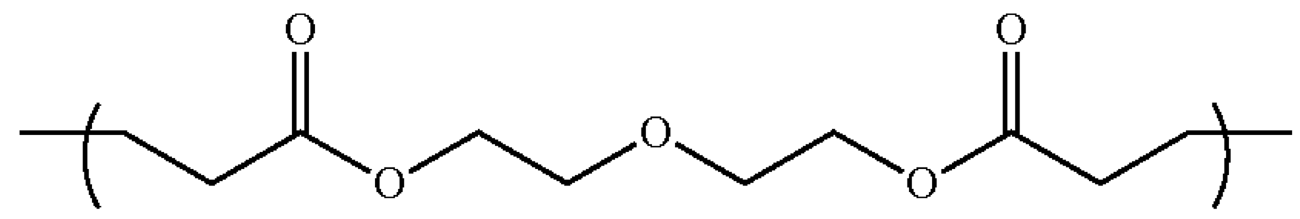
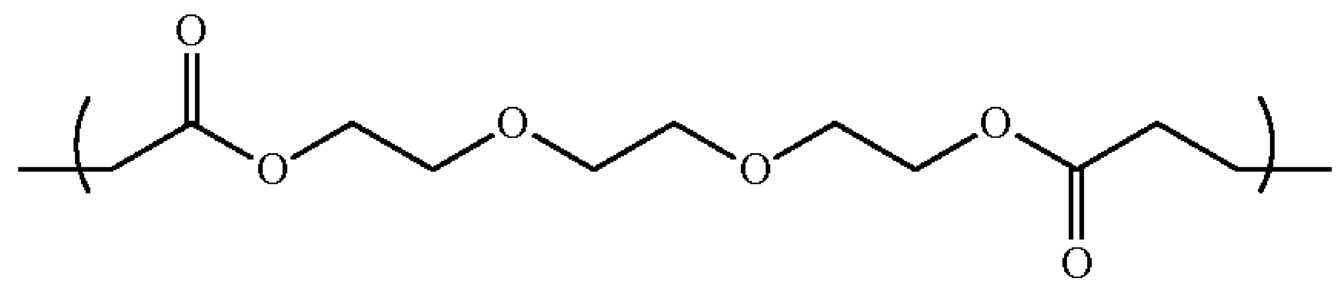
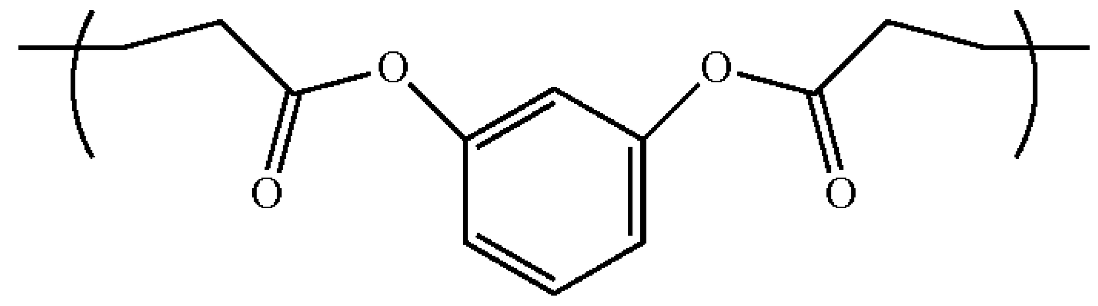
D'



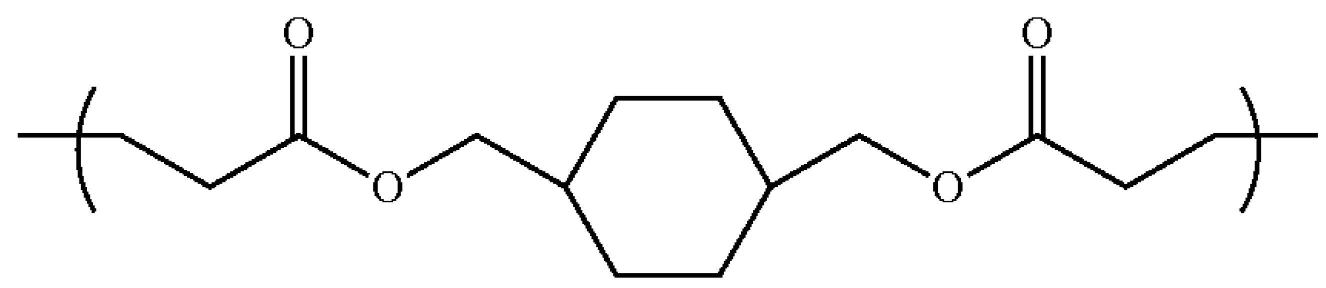
E'



F'



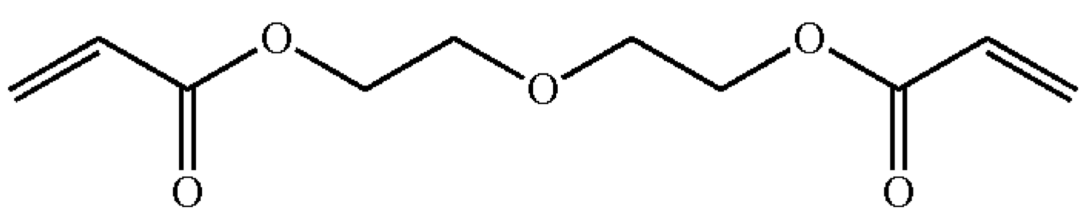
G'



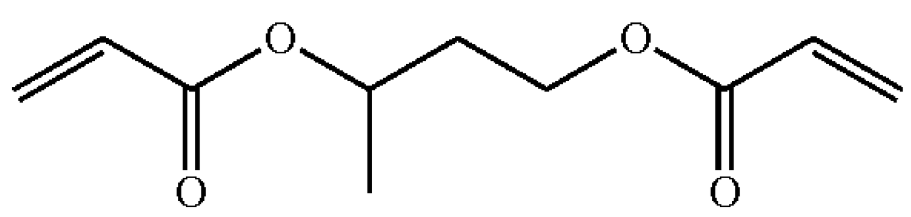
In certain embodiments, the polymer comprises the bis(acrylate ester) G'.

In another embodiment, the bis(acrylate ester) unit in the inventive polymer is chosen from the following group of bis(acrylate ester) units (A-PP):

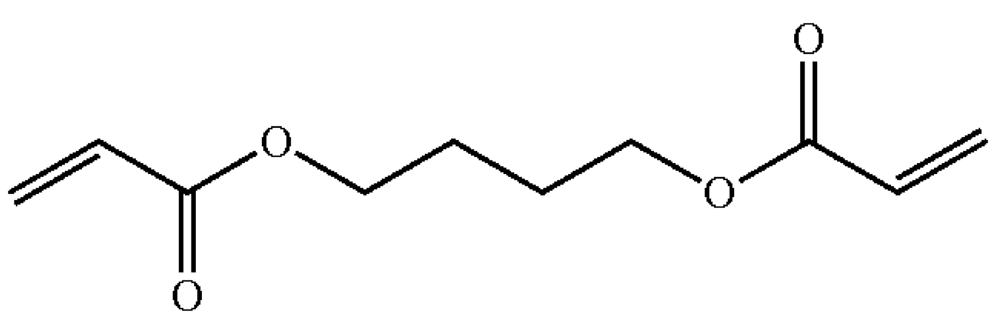
A



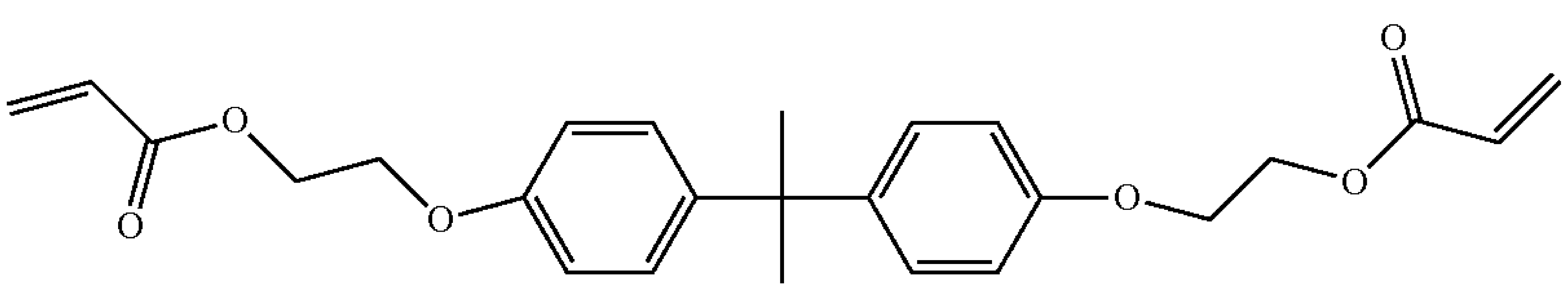
B



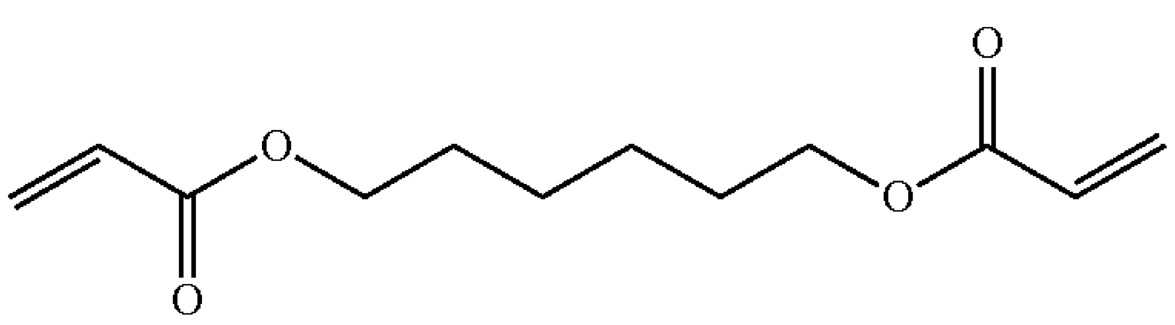
C



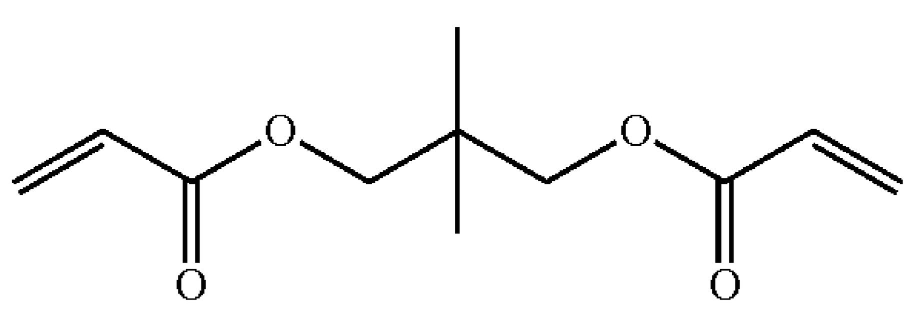
D



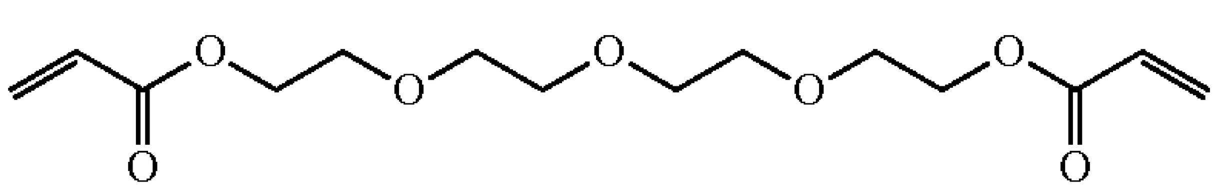
E



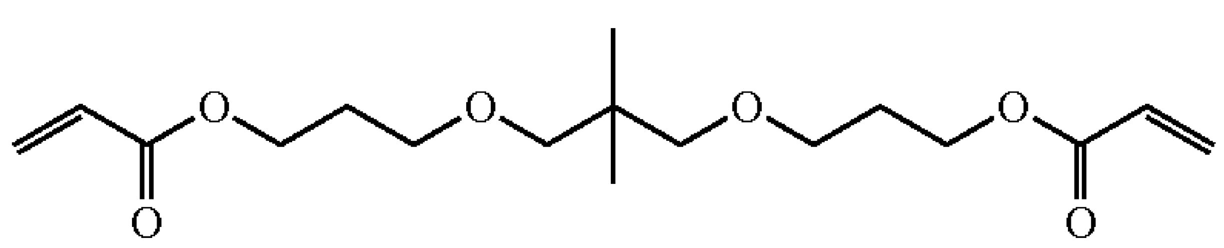
F



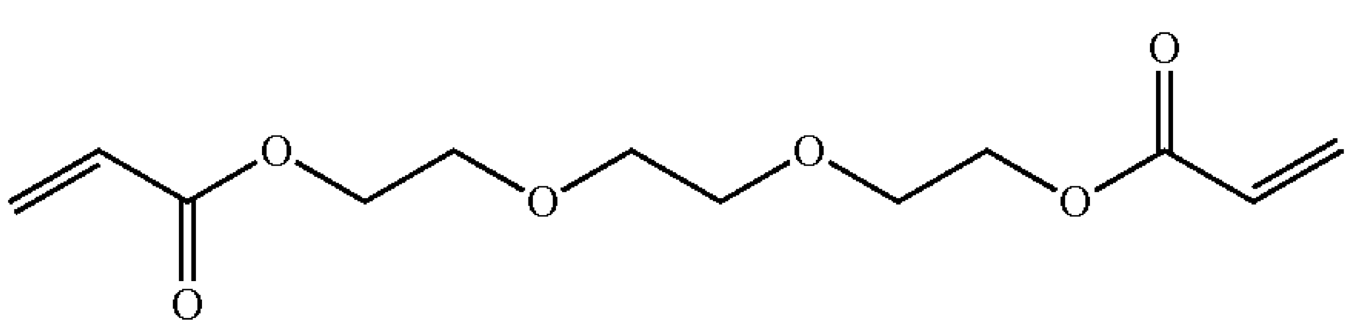
J



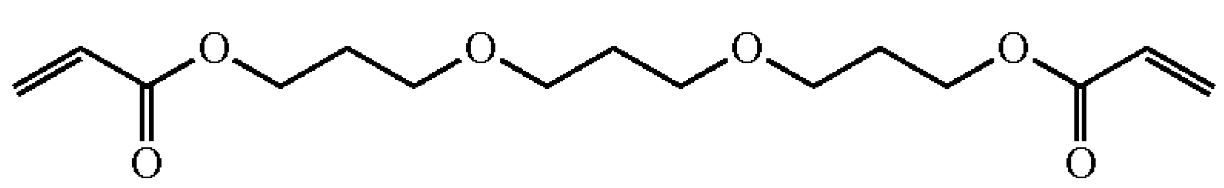
K



L



M



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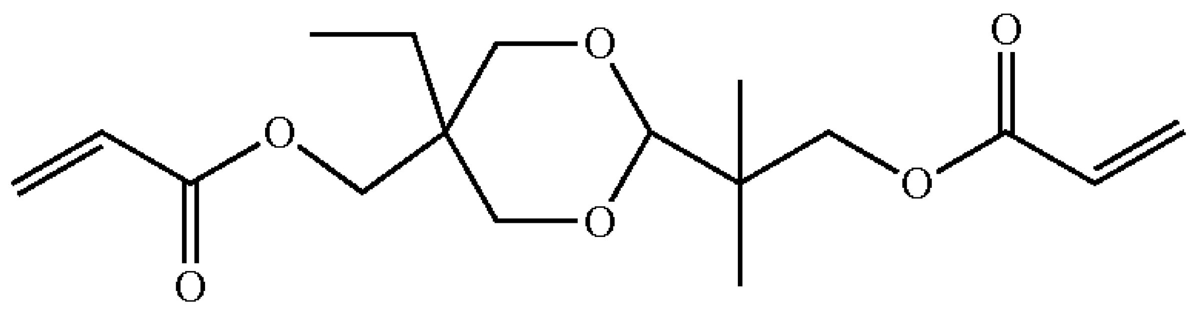
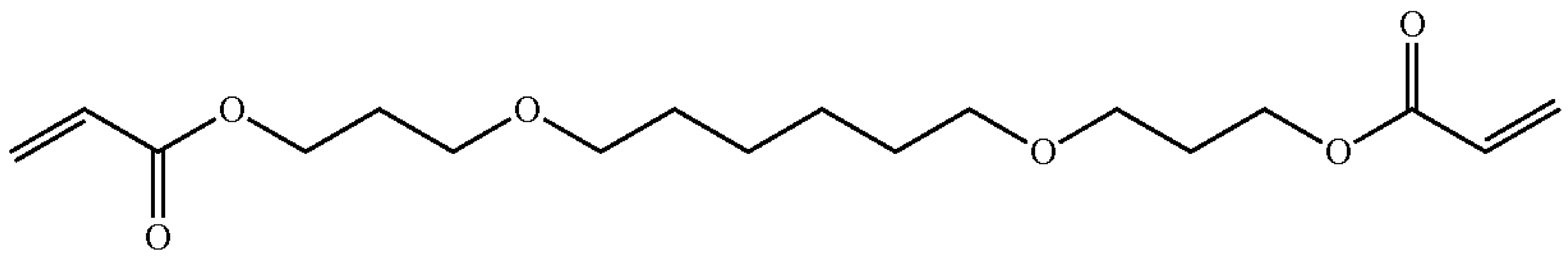
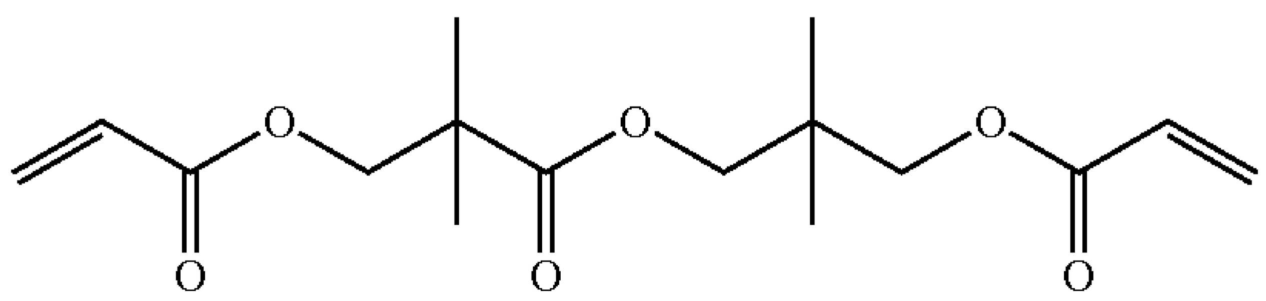


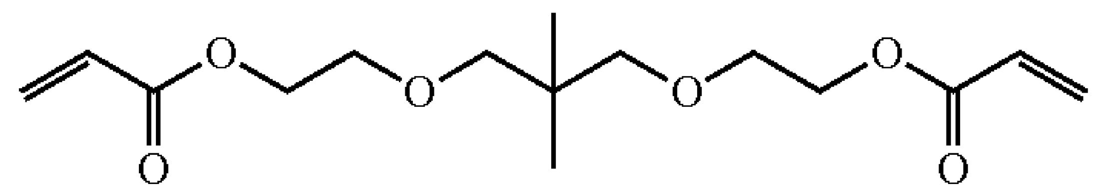
O



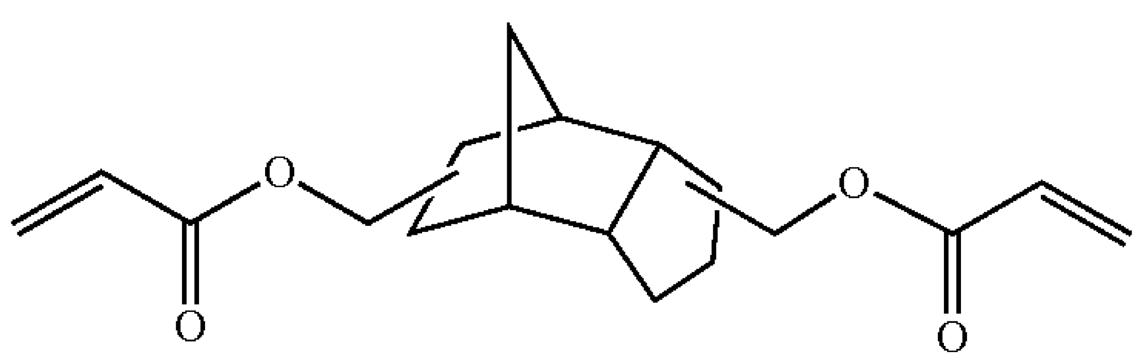
P



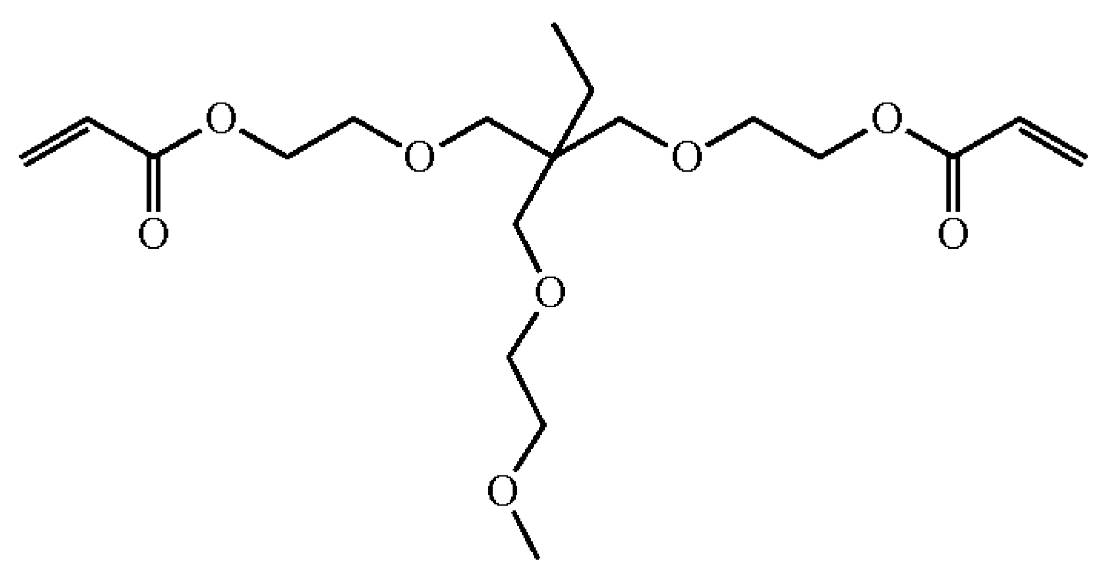
Q



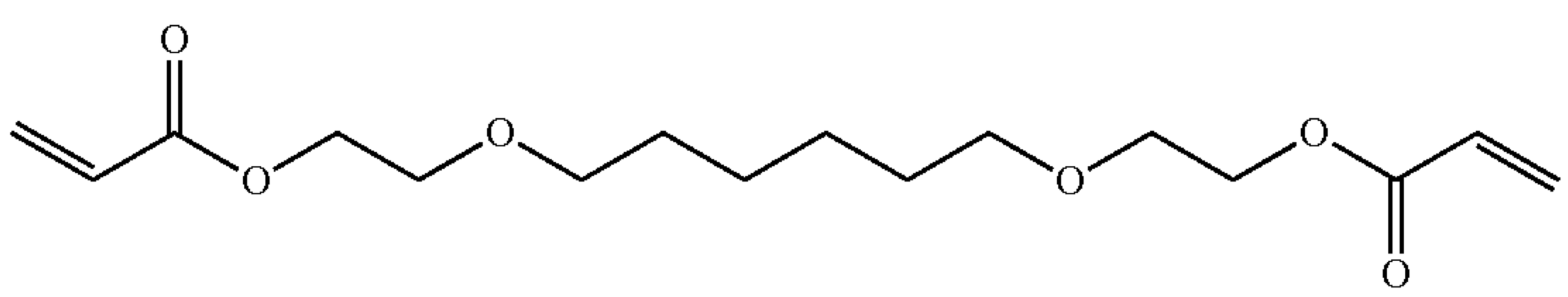
R



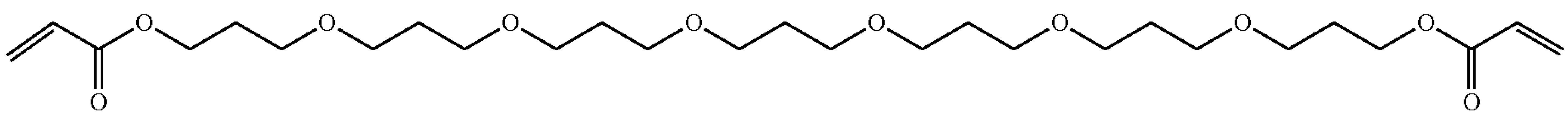
S



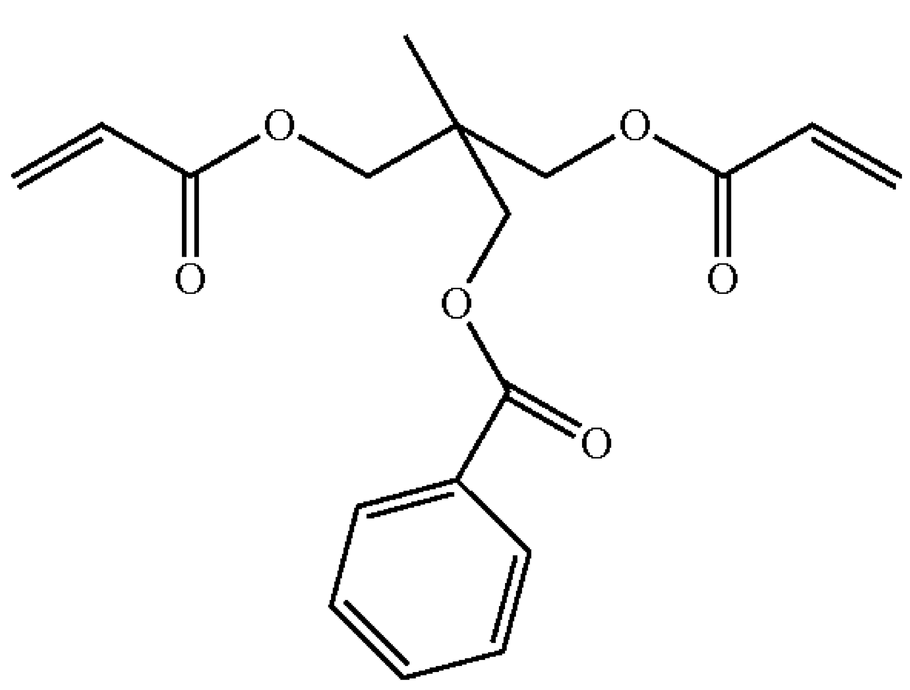
T



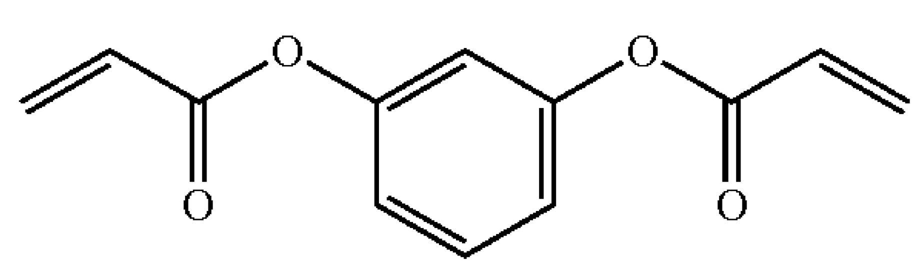
U



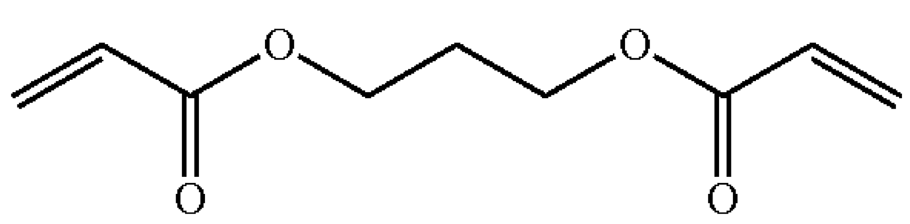
Z



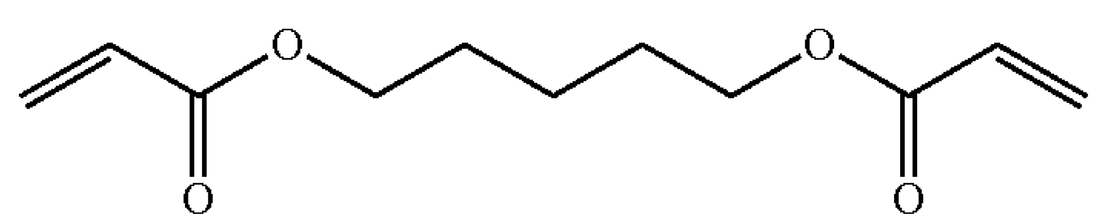
AA



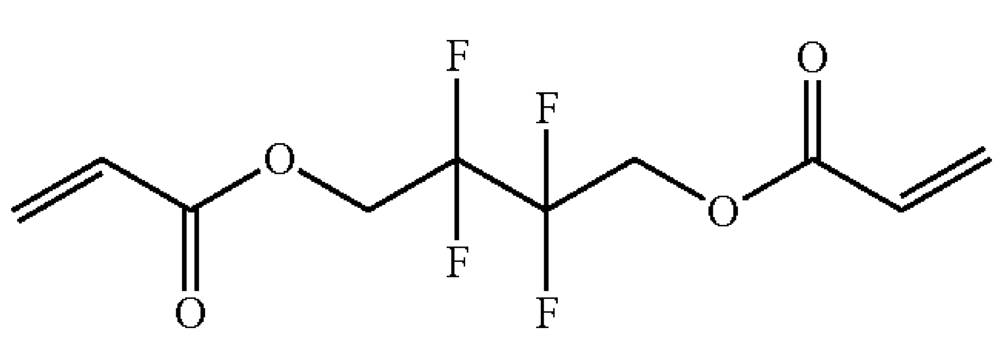
BB



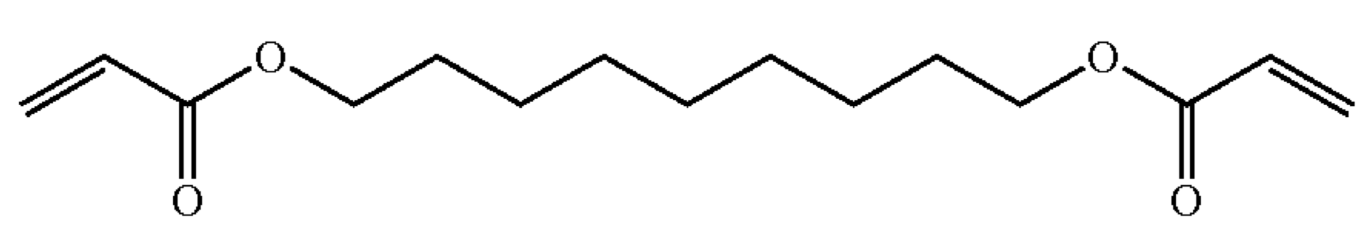
II



JJ



KK



LL

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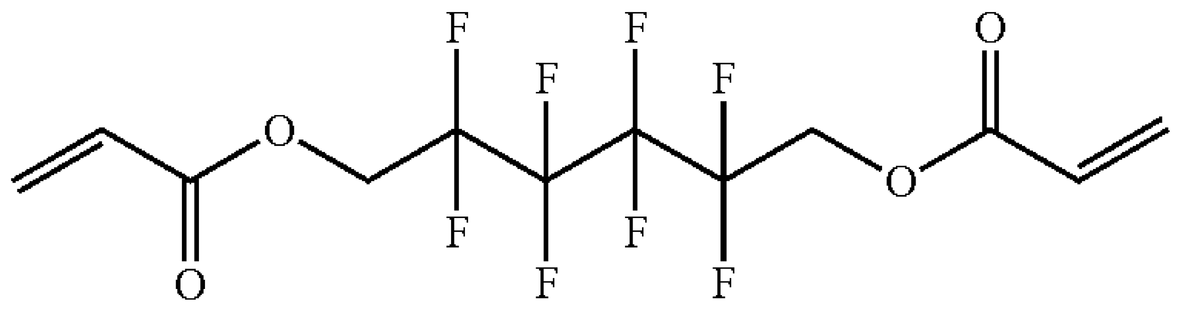
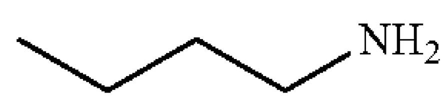
PP



Particularly preferred bis(acrylate esters) in this group include E, F, M, U, JJ, KK, LL, C, and D.

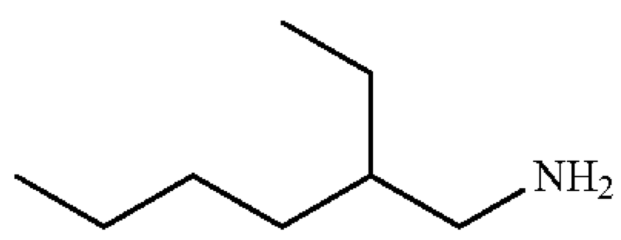
In another embodiment, the amine in the inventive polymer is chosen from the following group of amines (1'-20'):

1′

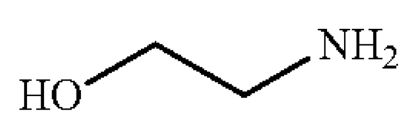


2′

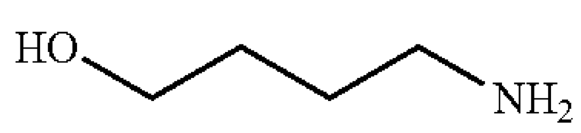
3′



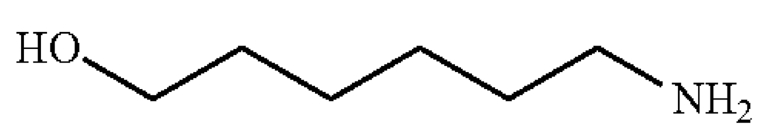
4′



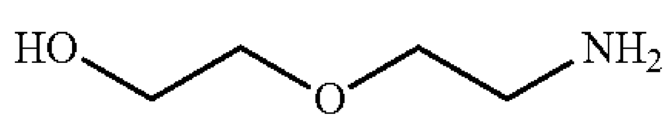
5′



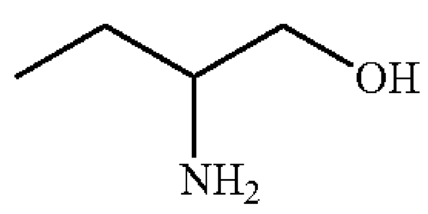
6′



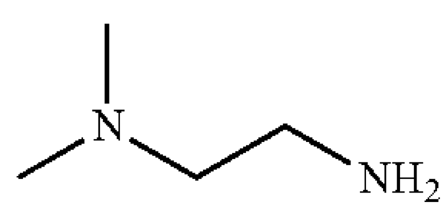
7′



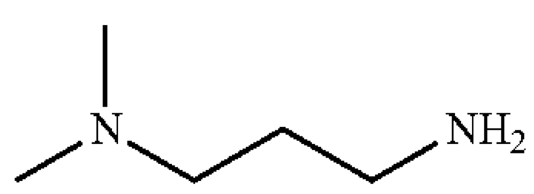
8′



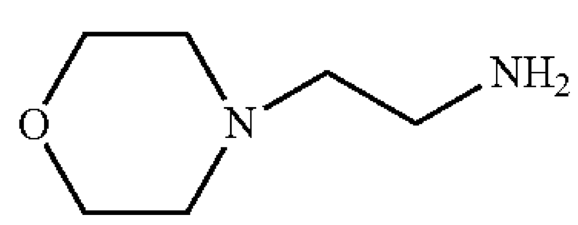
9′



10′



11′



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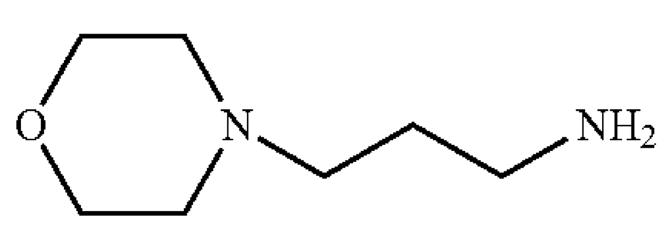

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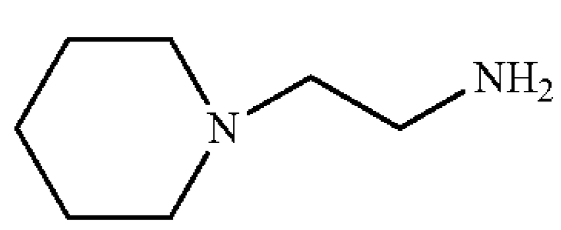

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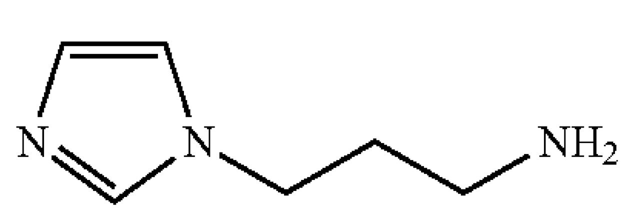

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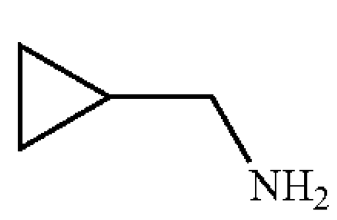

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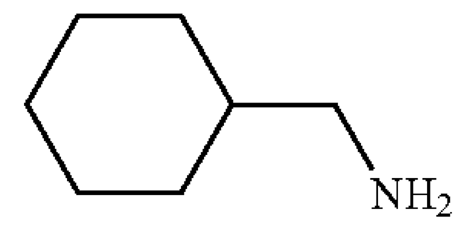
16′



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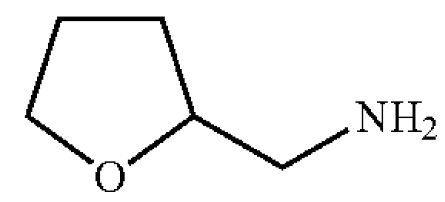

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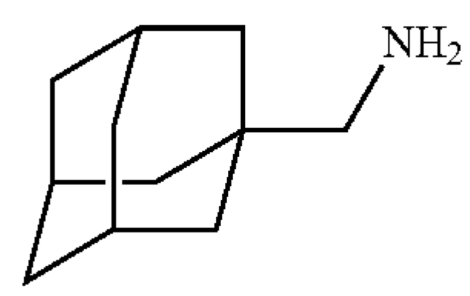

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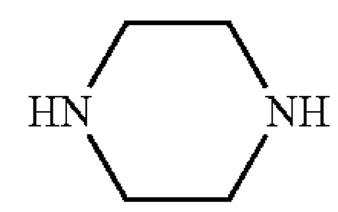
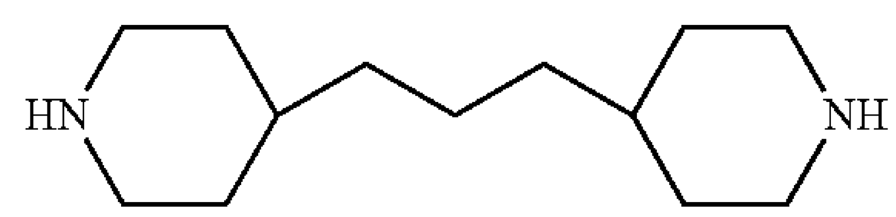


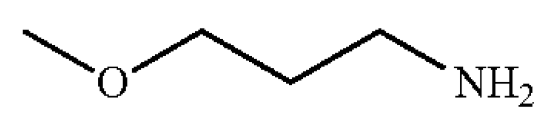
20′



In certain embodiments, the polymer comprises the amine 5'. In other embodiments, the polymer comprises amine 14'.

In another embodiment, the amine in the inventive polymer is chosen from the following group of amines (1-94):

1



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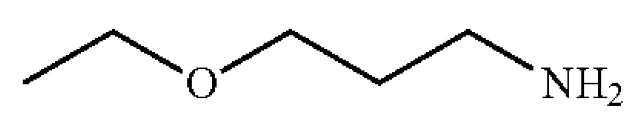

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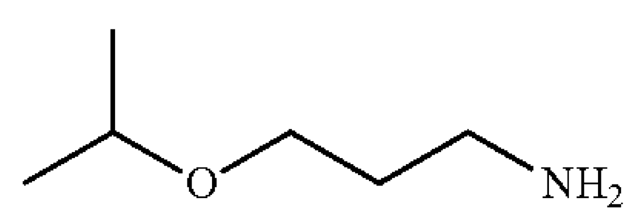

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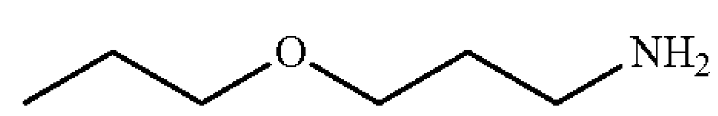

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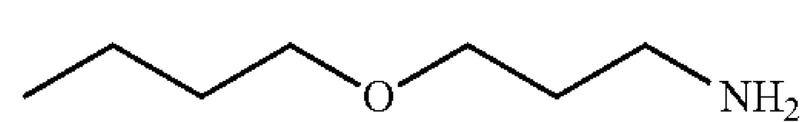

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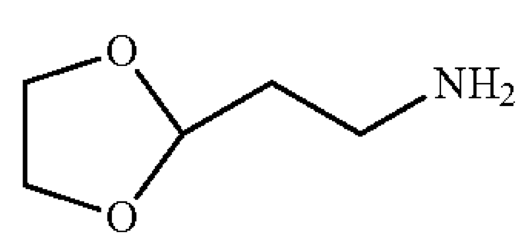

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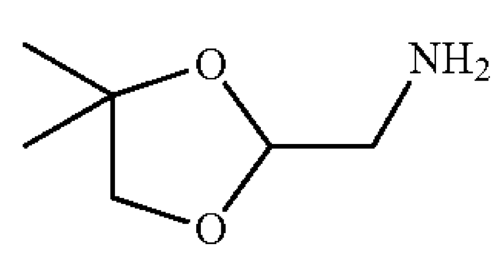

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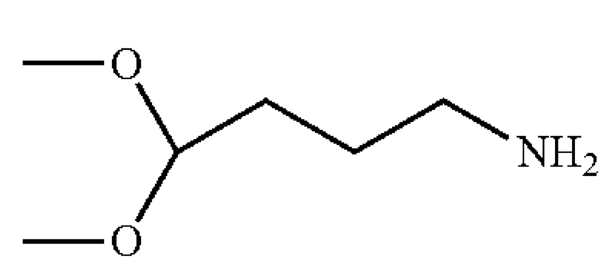

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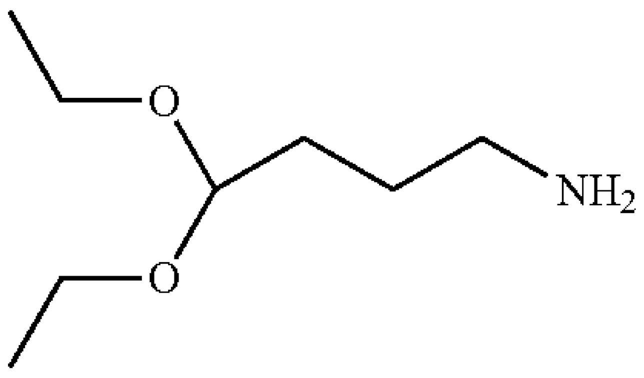
9

10

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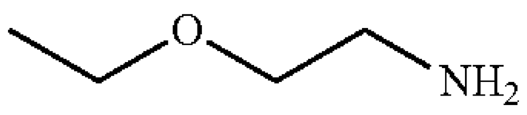
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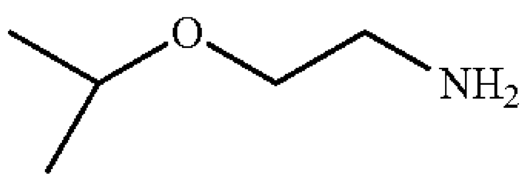
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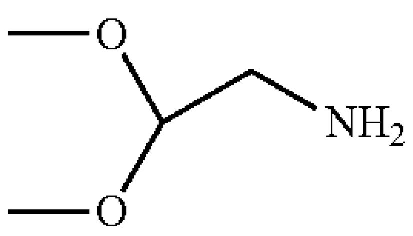
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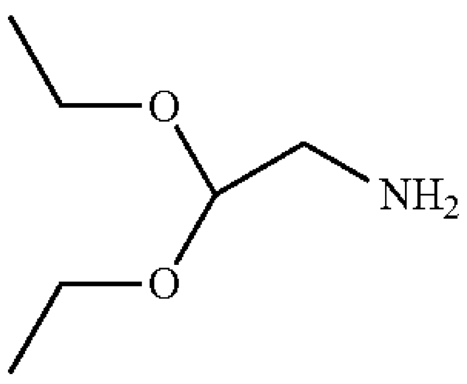
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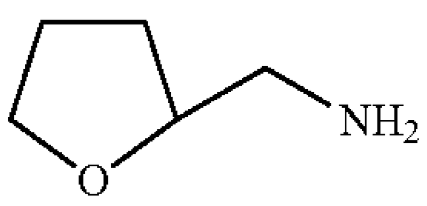
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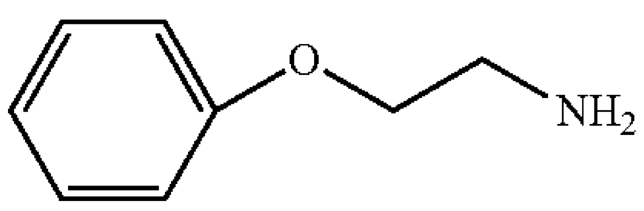
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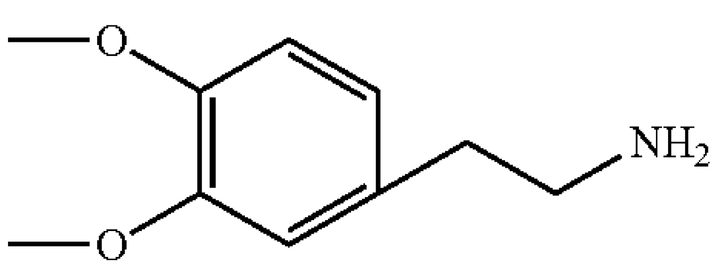
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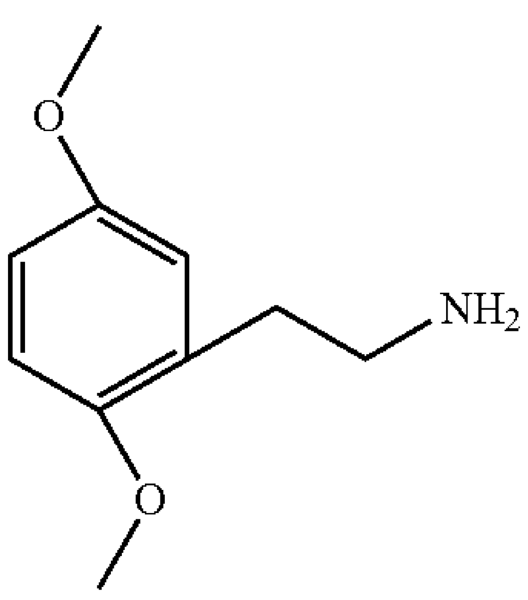
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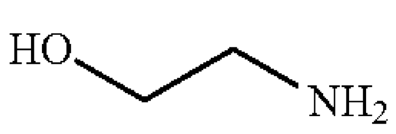
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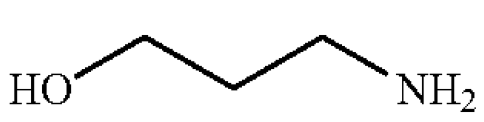
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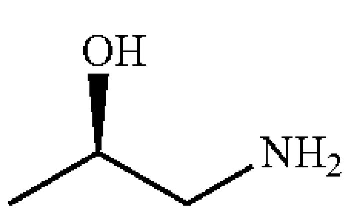
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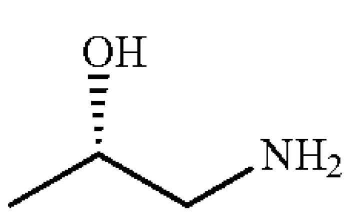



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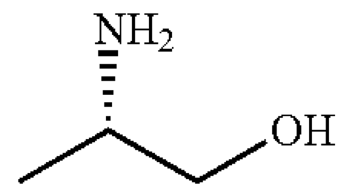
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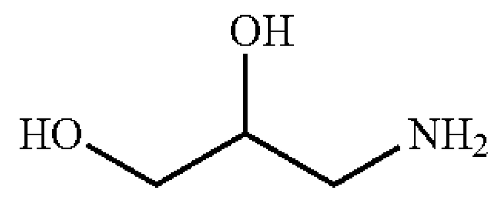
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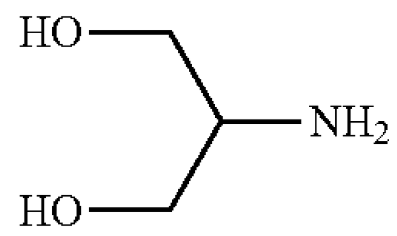
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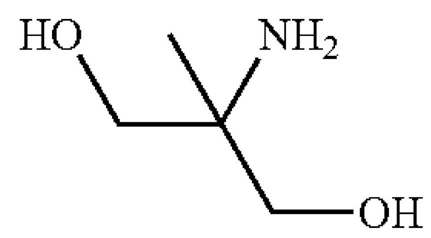
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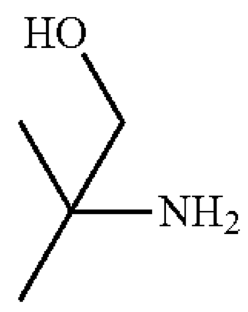
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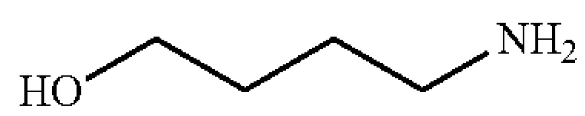
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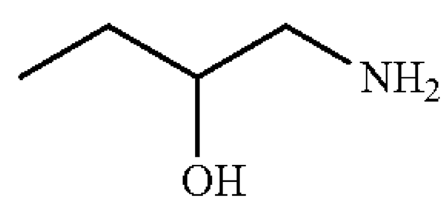
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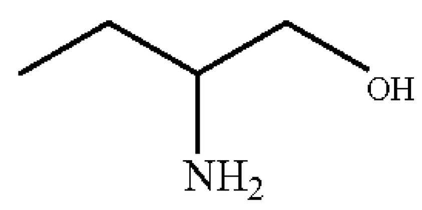
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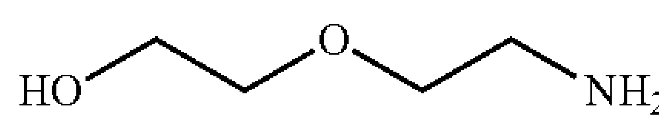
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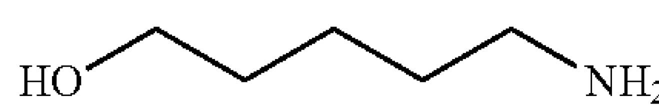
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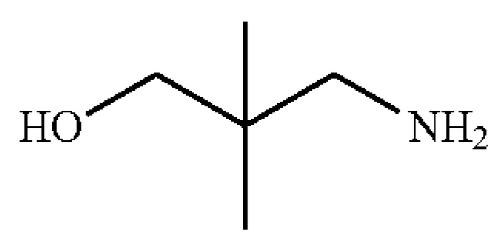
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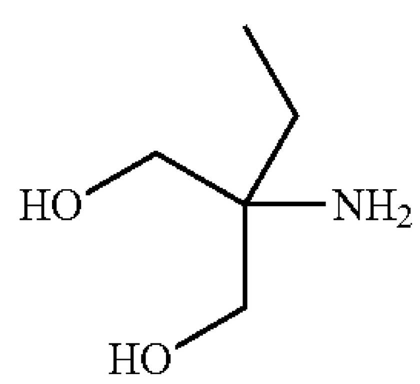
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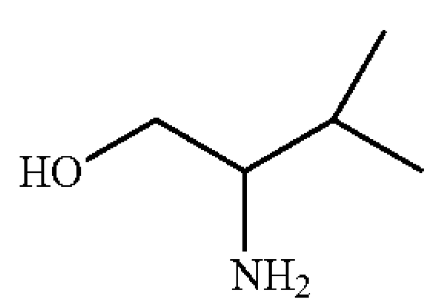
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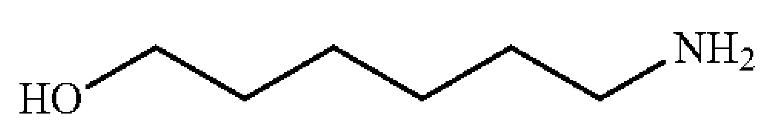
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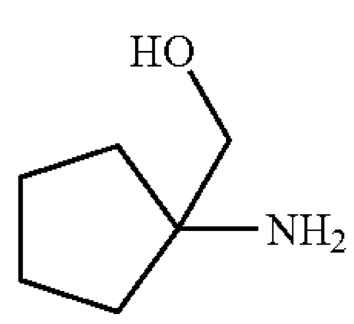
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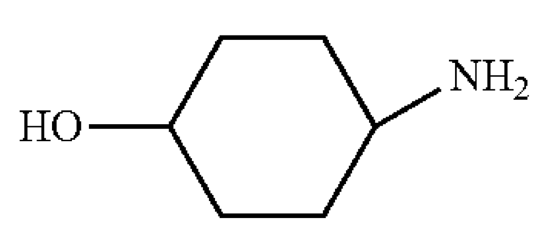

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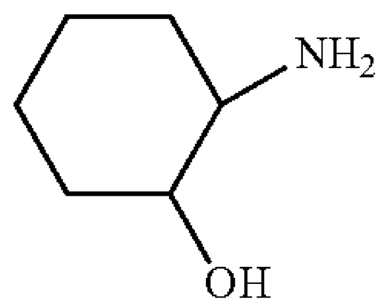

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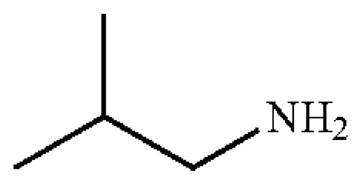

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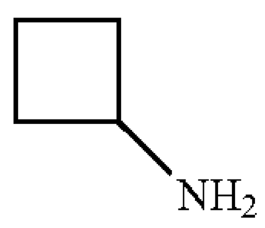

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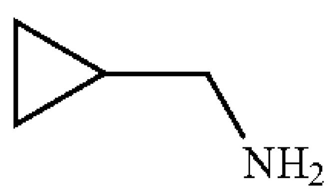

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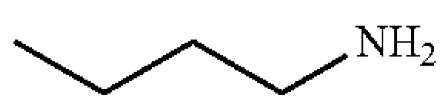

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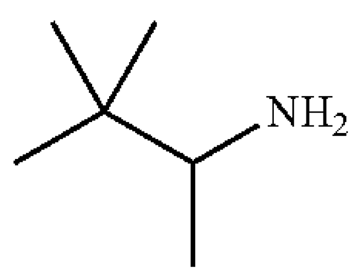

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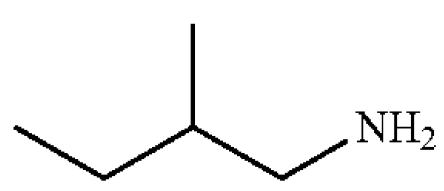

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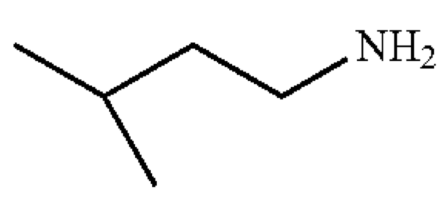

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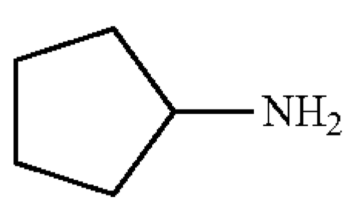

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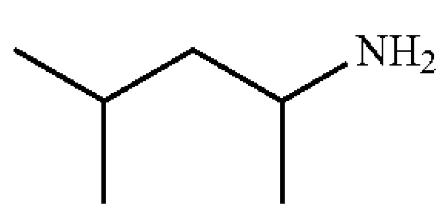

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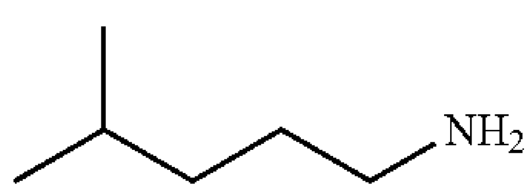

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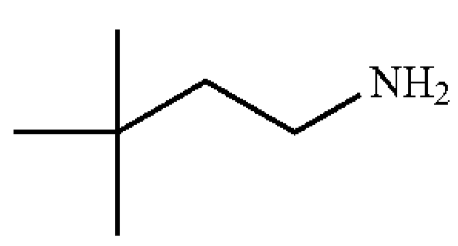

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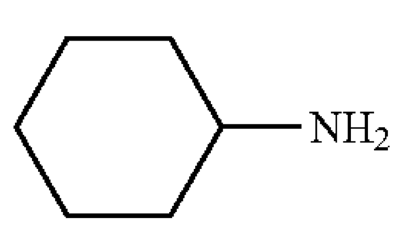

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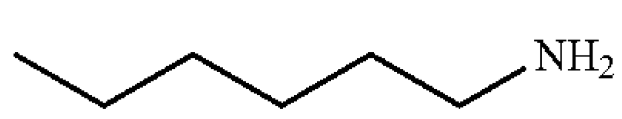

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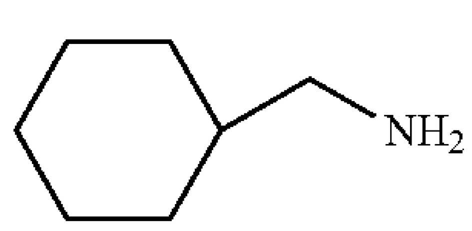

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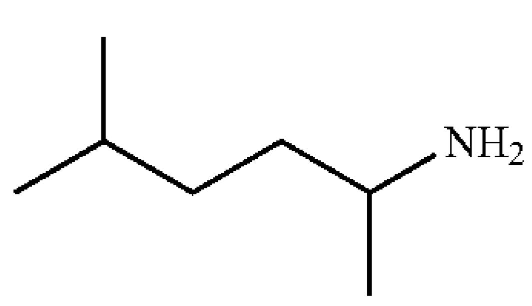



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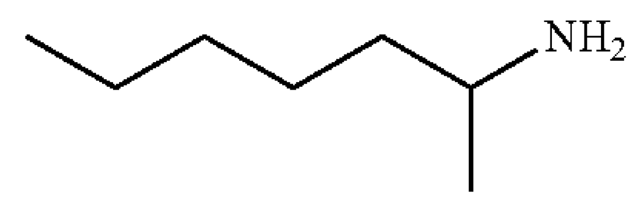

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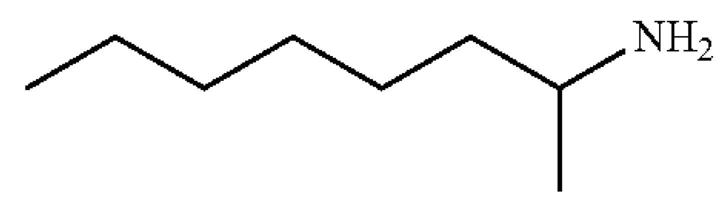

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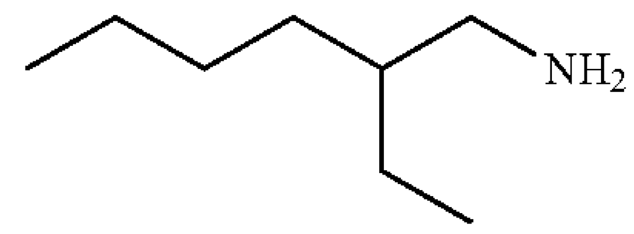

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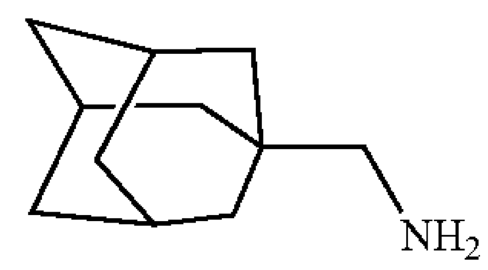

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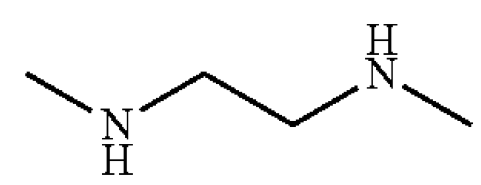

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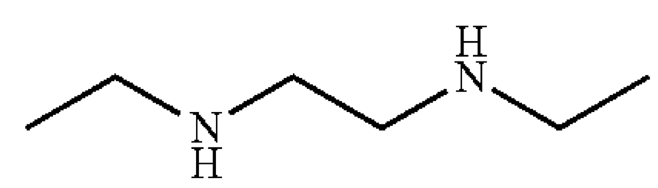

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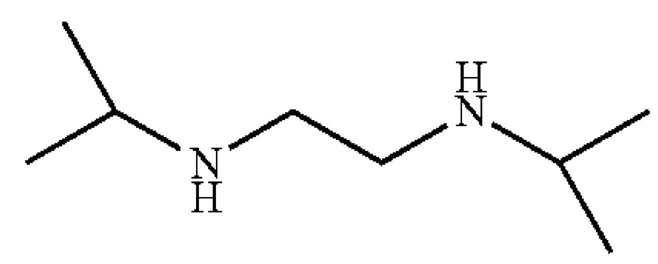

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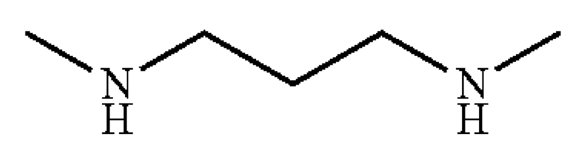

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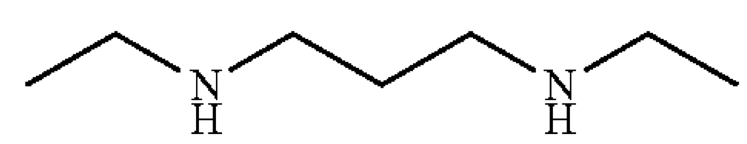

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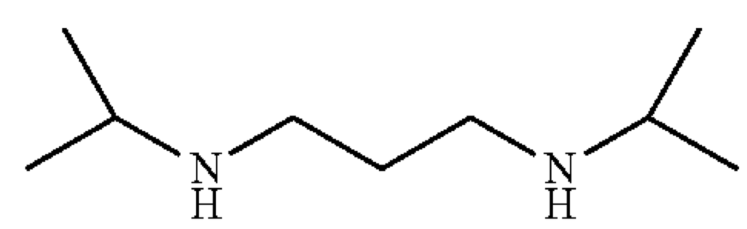

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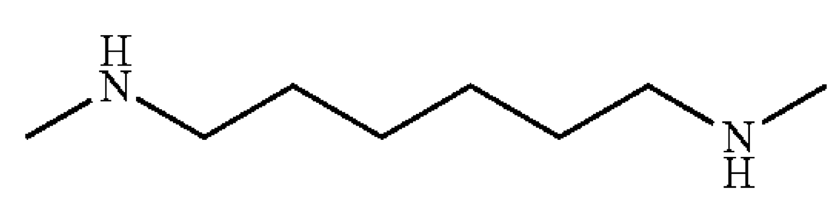

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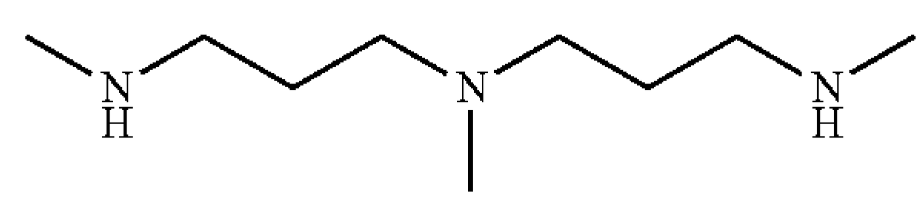

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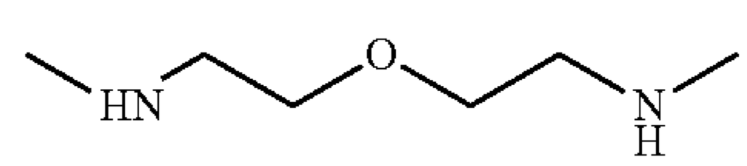

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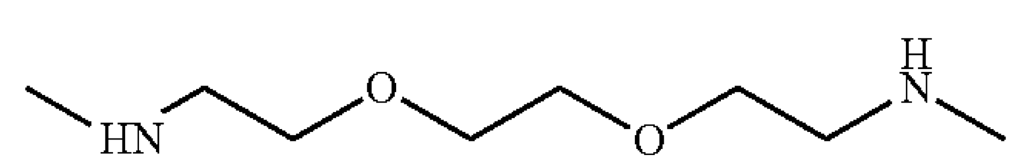

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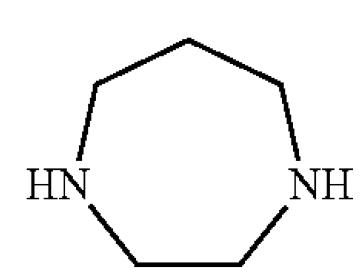

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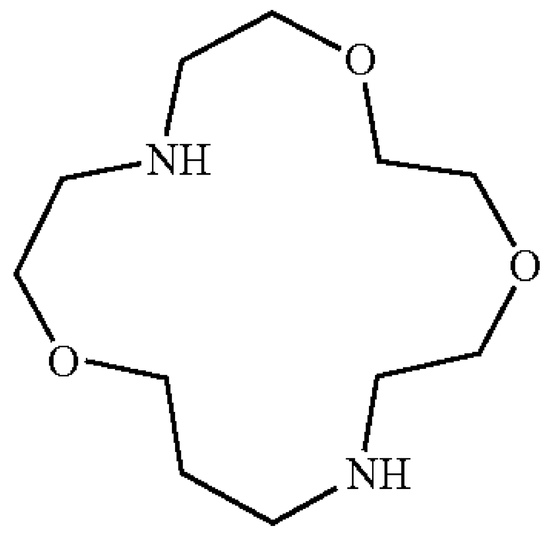

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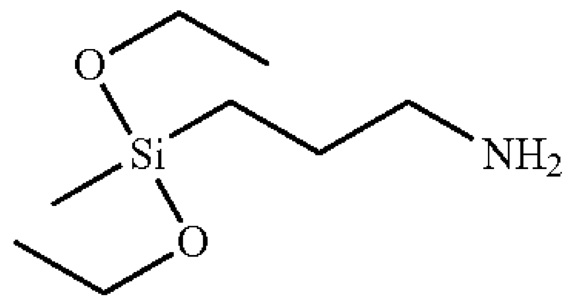

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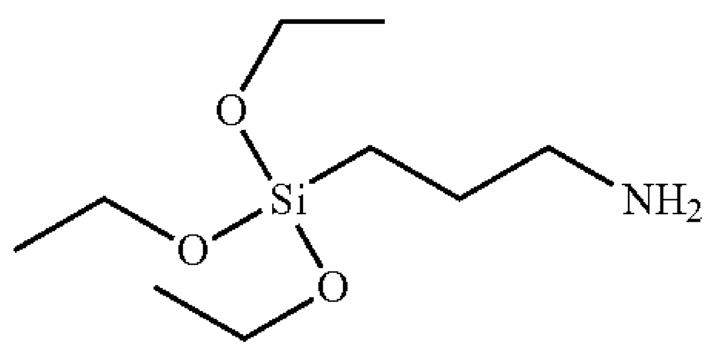

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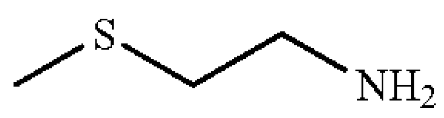

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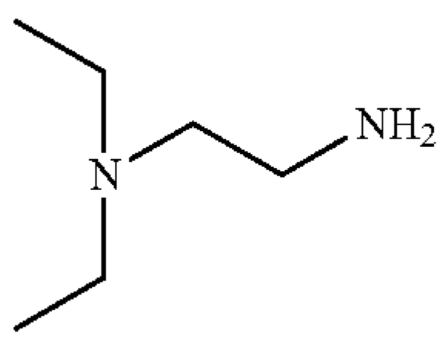

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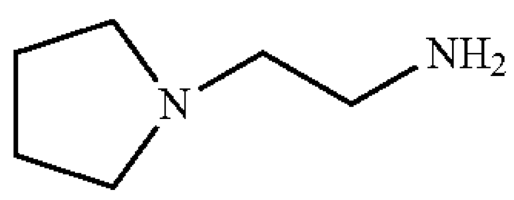

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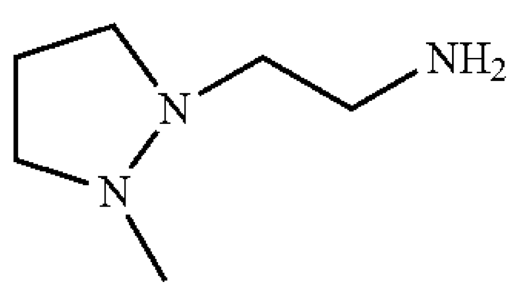

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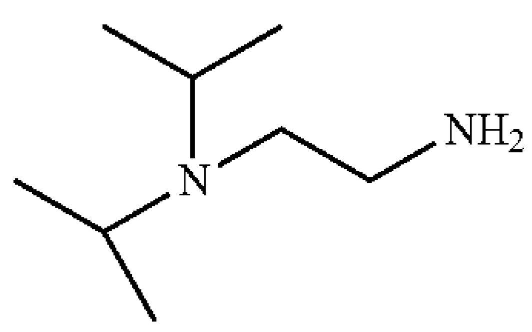

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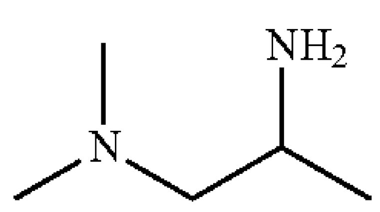

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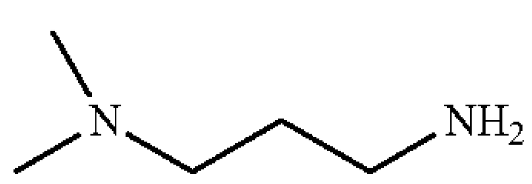



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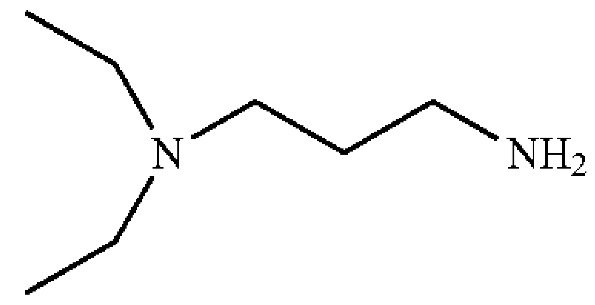

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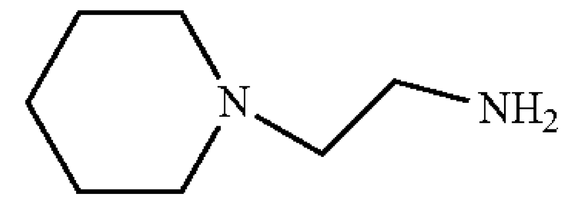

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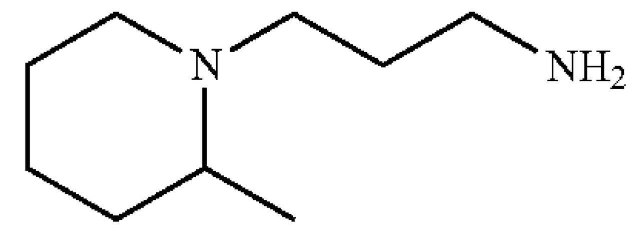

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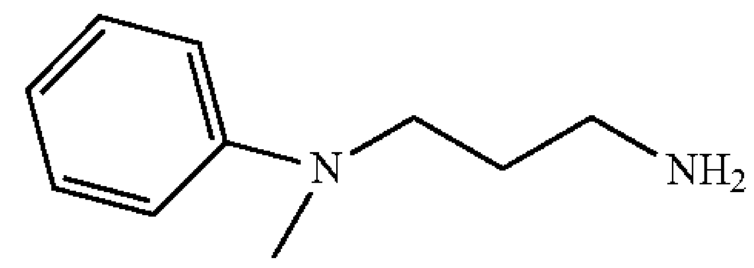

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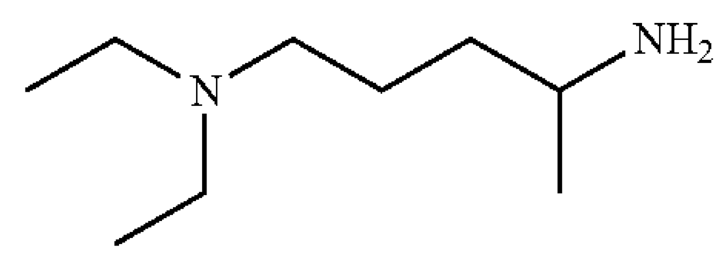

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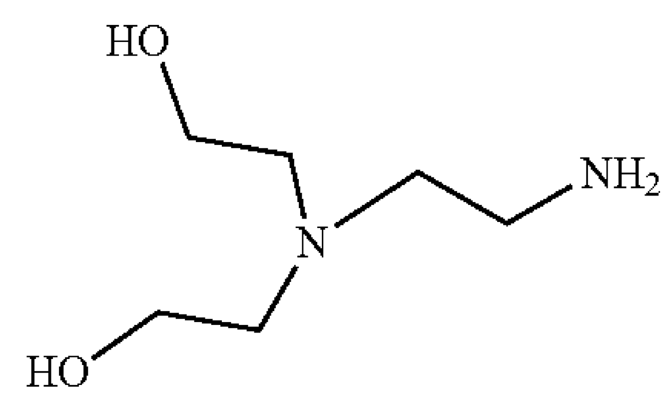

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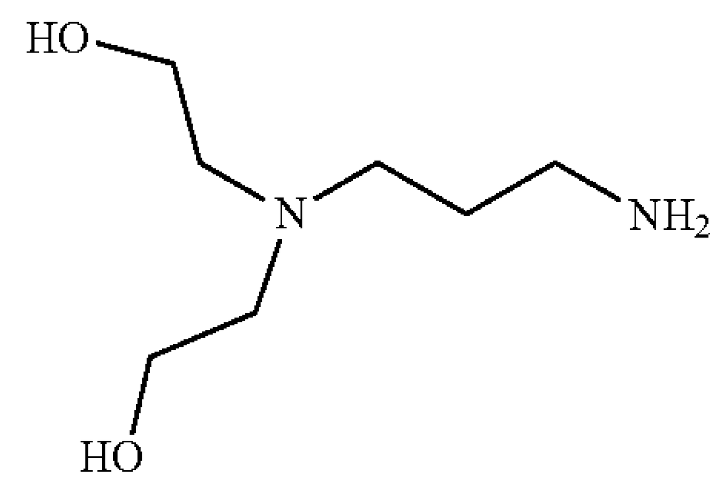

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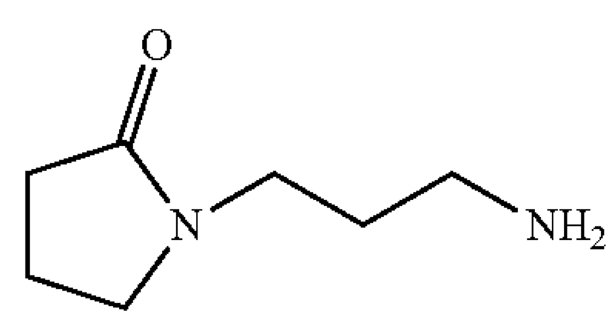

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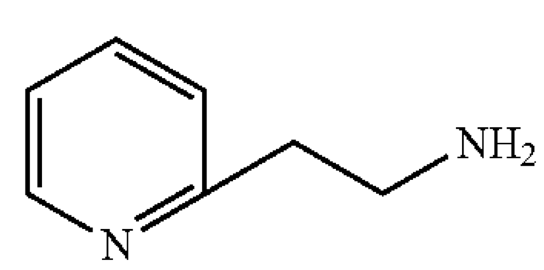

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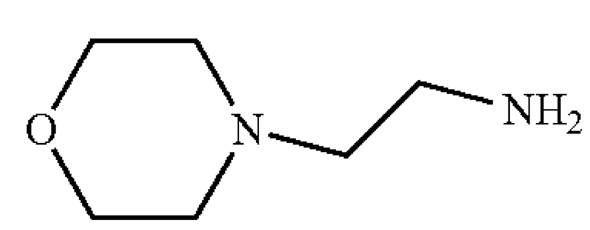

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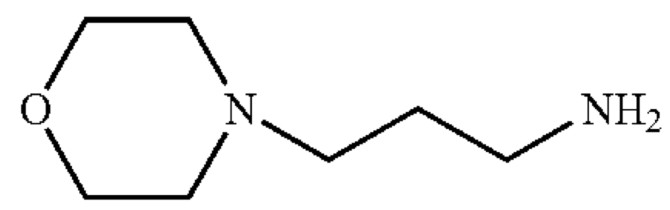

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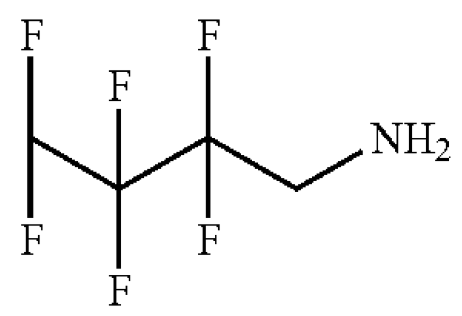

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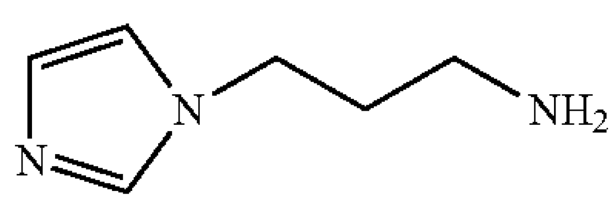
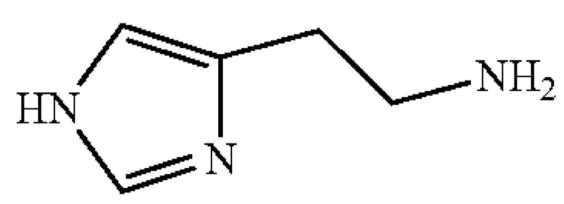


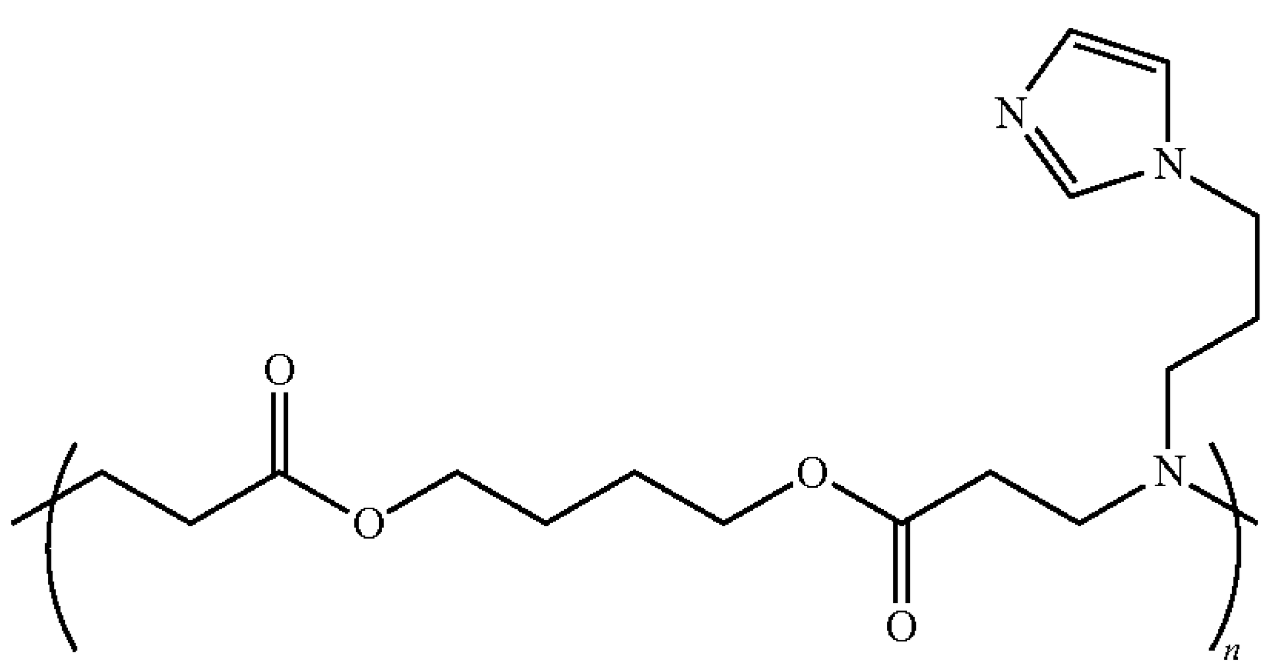
94



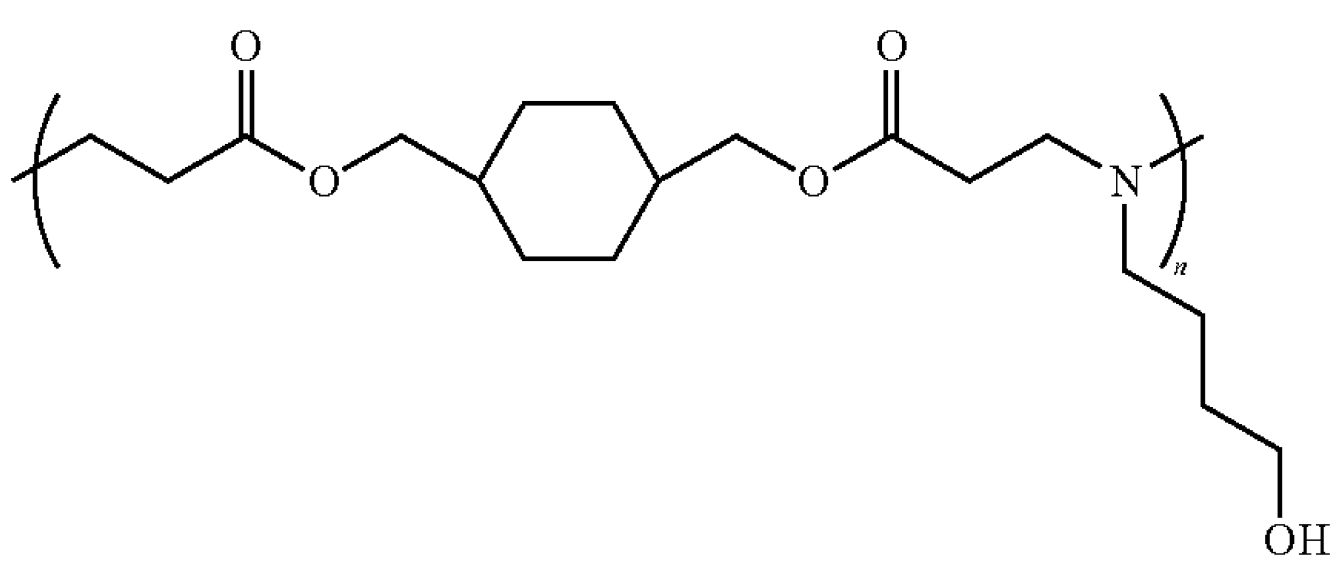
In certain embodiments, the polymers include amines 6, 17, 20, 28, 32, 36, 60, 61, 86, 89, or 94.

Particular examples of the polymers of the present invention include:

B′14′



G′5′





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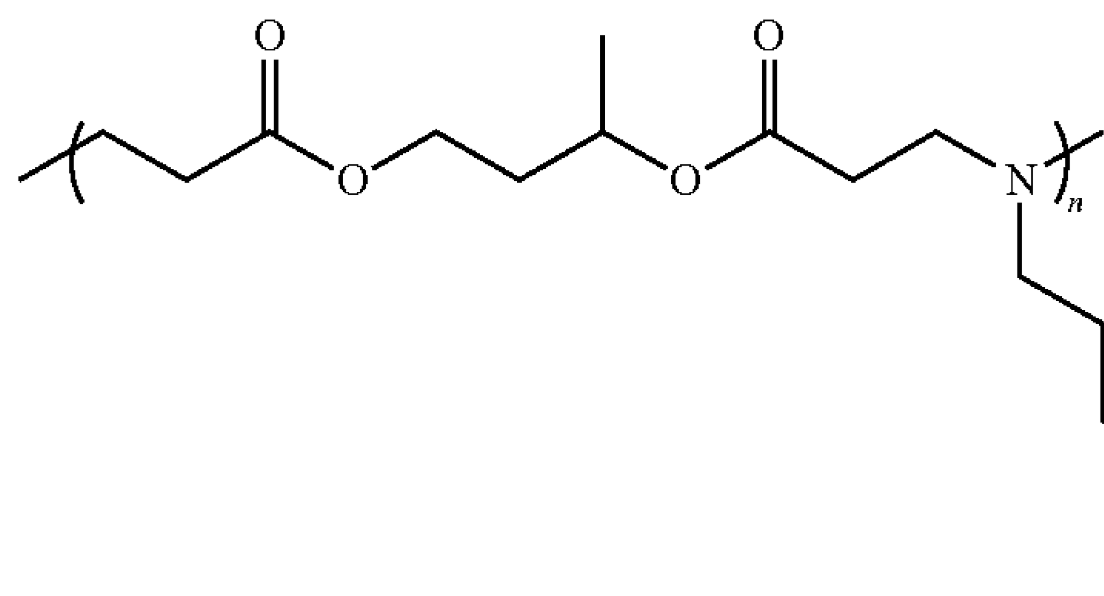
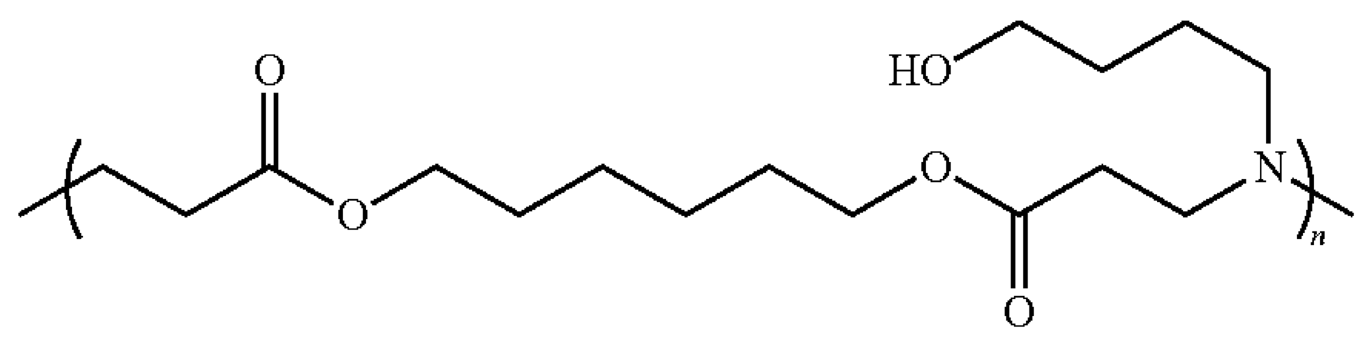
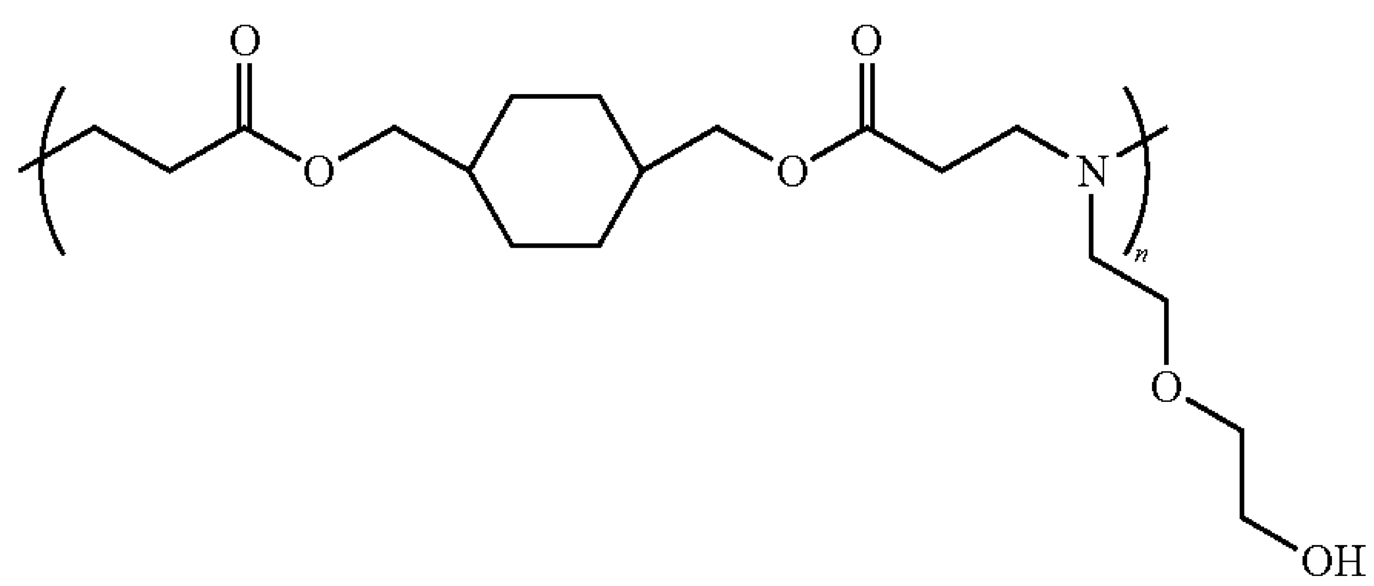
A′14′



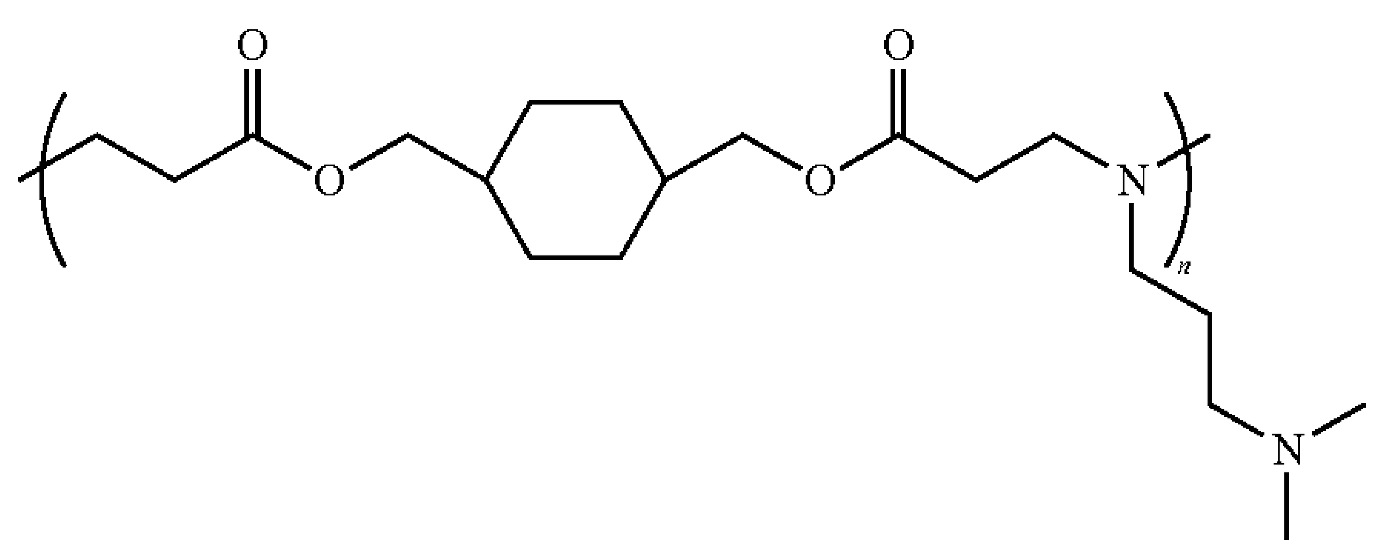
C′5′



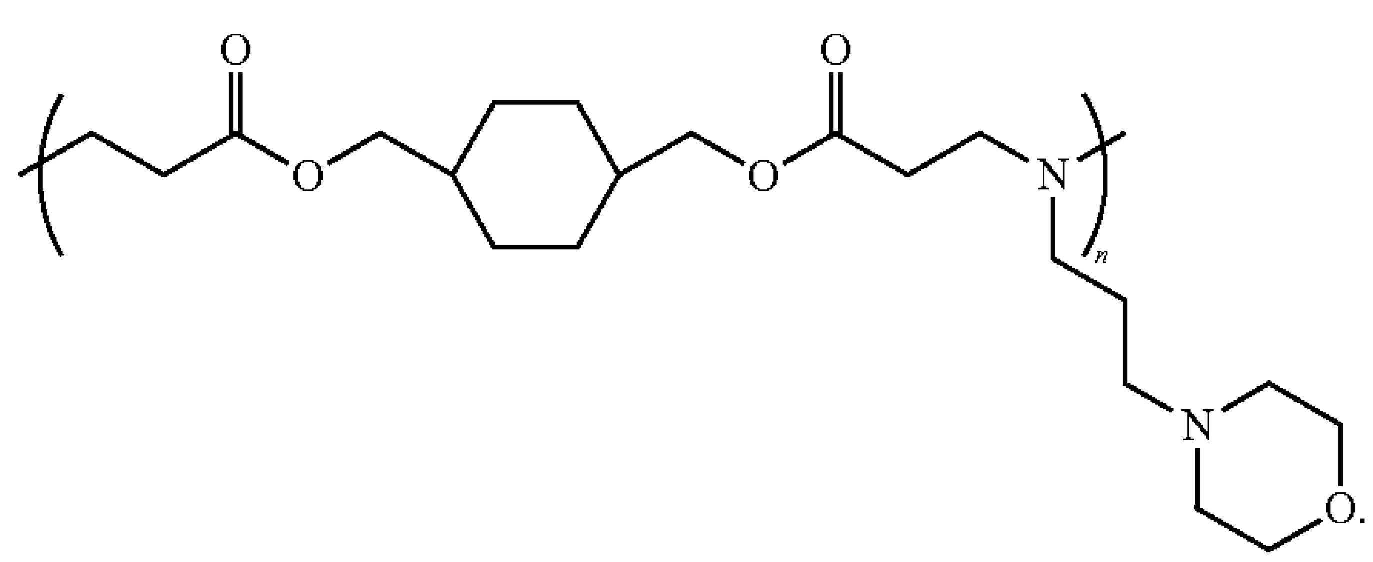
G′7′



G′10′

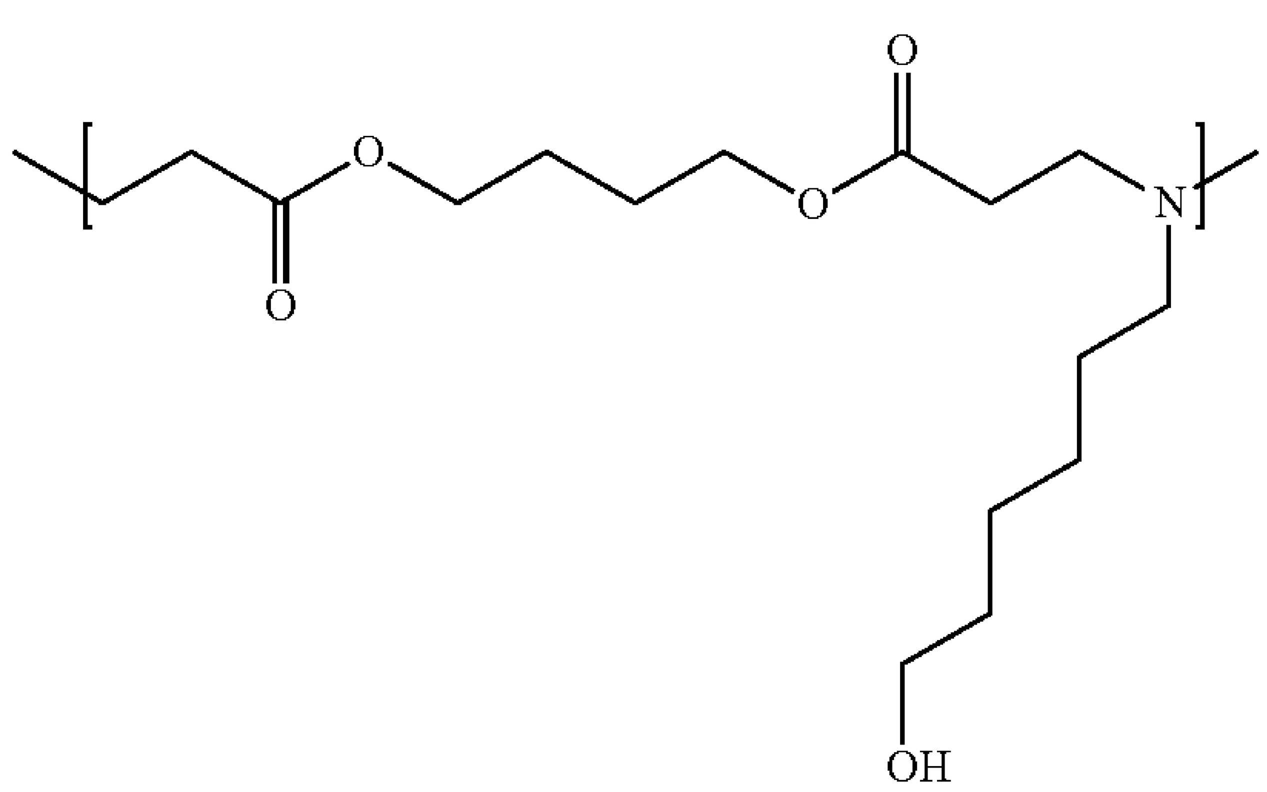


G′12′



Other particularly useful poly(beta-amino ester)s include:

C36



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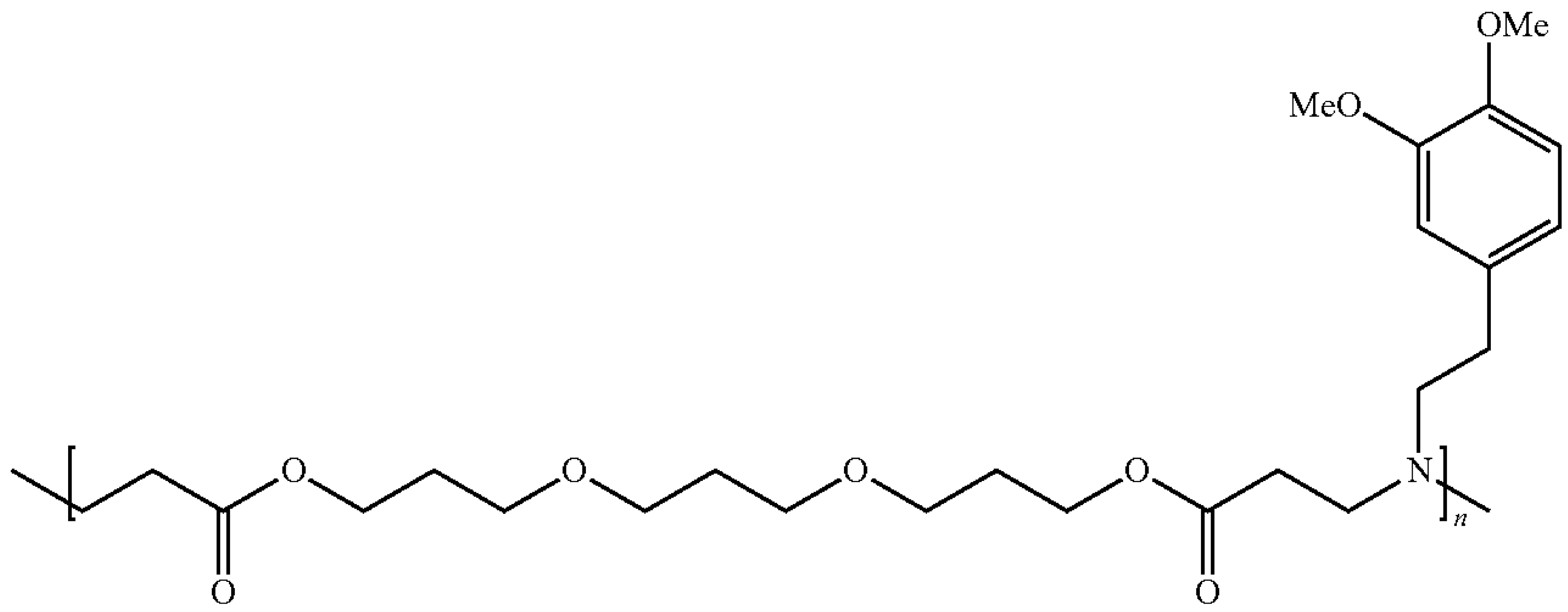
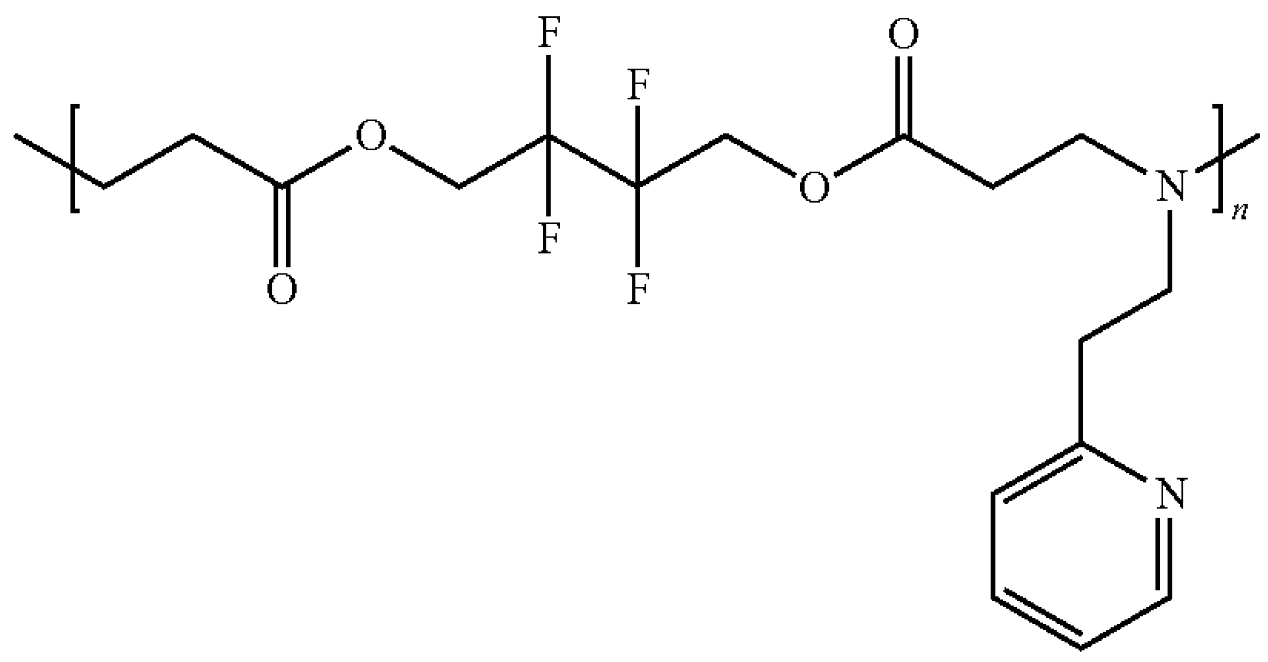
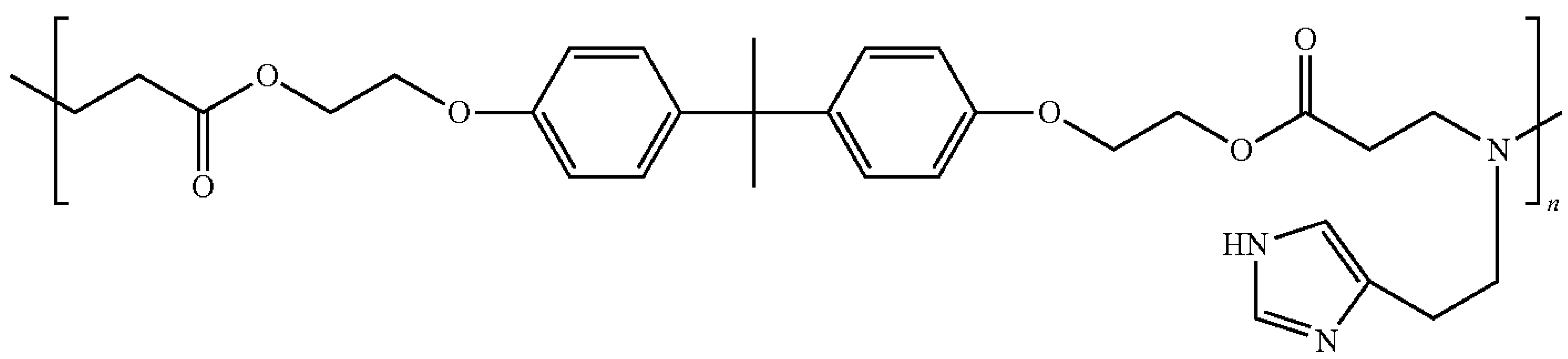
M17



KK89



D94



In a particularly preferred embodiment, one or both of the linkers A and B are polyethylene polymers. In another particularly preferred embodiment, one or both of the linkers A and B are polyethylene glycol polymers. Other biocompatible, biodegradable polymers may be used as one or both of the linkers A and B.

In certain preferred embodiments, the polymers of the present invention are amine-terminated. In other embodiments, the polymers of the present invention are acrylate-terminated.

In certain embodiments, the average molecular weight of the polymers of the present invention range from 1,000 g/mol to 50,000 g/mol, preferably from 2,000 g/mol to 40,000 g/mol, more preferably from 5,000 g/mol to 20,000 g/mol, and even more preferably from 10,000 g/mol to 17,000 g/mol. Since the polymers of the present invention are prepared by a step polymerization, a broad, statistical distribution of chain lengths is typically obtained. In certain embodiments, the distribution of molecular weights in a polymer sample is narrowed by purification and isolation steps known in the art. In other embodiments, the polymer mixture may be a blend of polymers of different molecular weights.

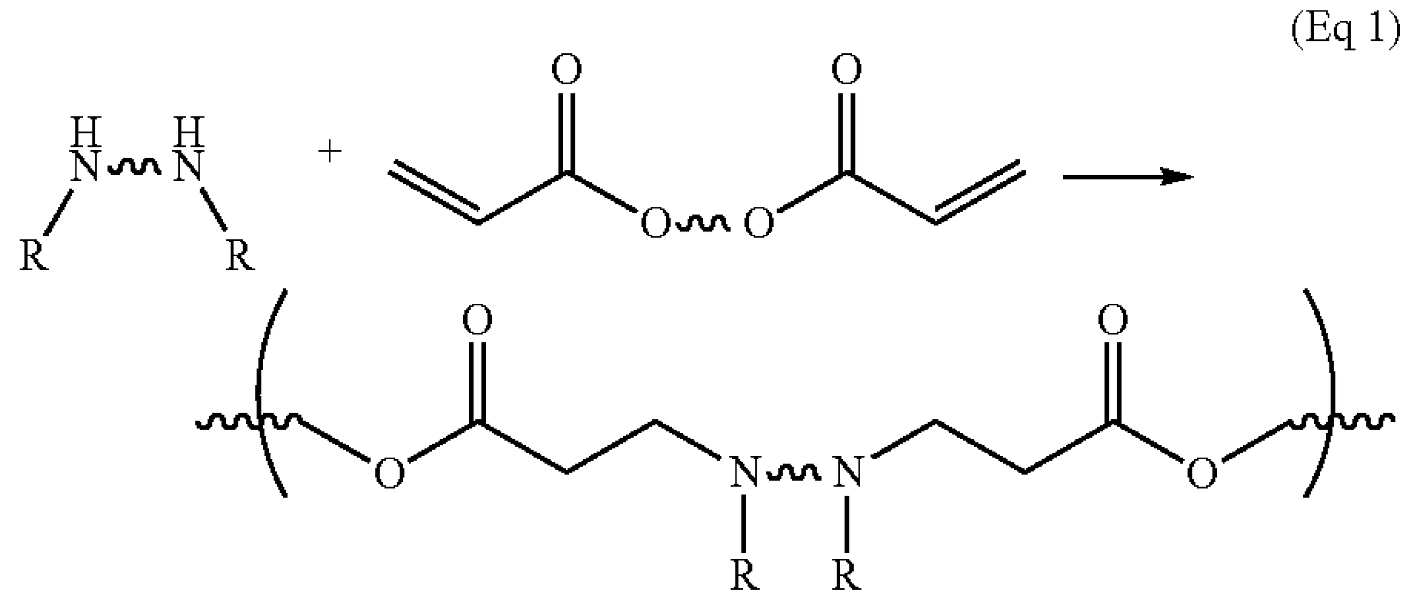
In another particularly preferred embodiment, the polymer of the present invention is a co-polymer wherein one of the repeating units is a poly(β-amino ester) of the present invention. Other repeating units to be used in the co-polymer include, but are not limited to, polyethylene, poly(glycolide-co-lactide) (PLGA), polyglycolic acid, polymethacrylate, etc.

Synthesis of Polymers

The inventive polymers may be prepared by any method known in the art. Preferably the polymers are prepared from commercially available starting materials. In another preferred embodiment, the polymers are prepared from easily and/or inexpensively prepared starting materials.

In a particularly preferred embodiment, the inventive polymer is prepared via the conjugate addition of bis(secondary amines) to bis(acrylate esters). This reaction scheme is shown below:

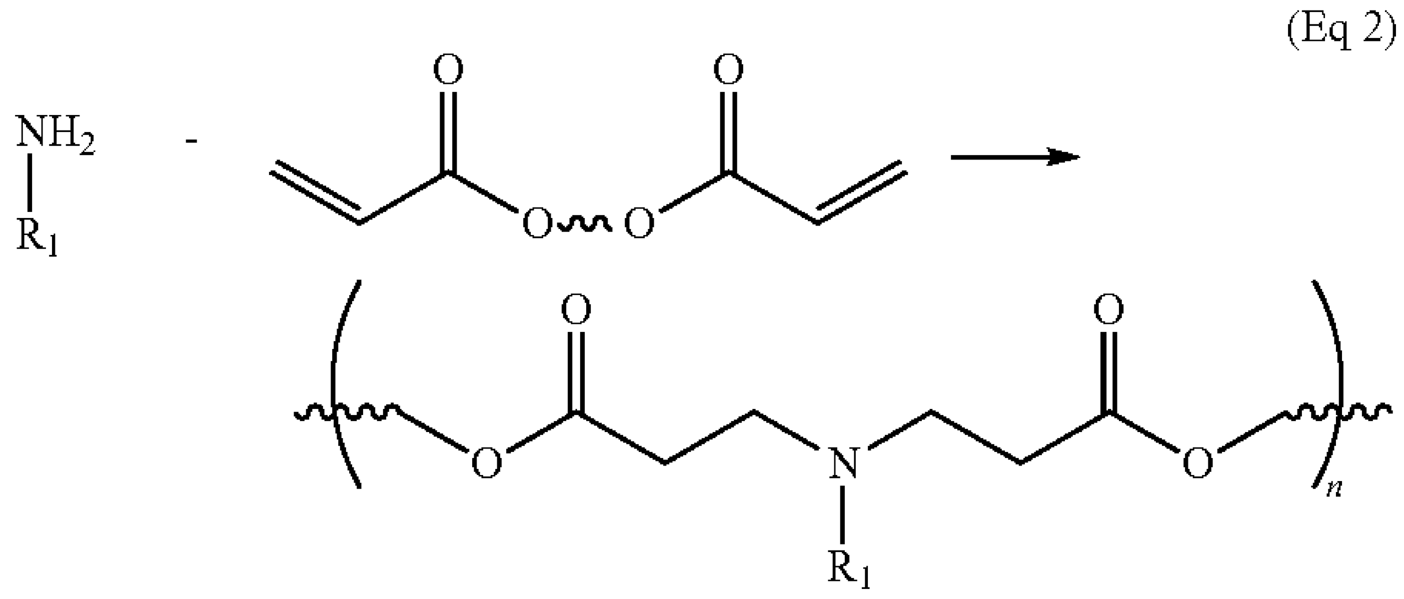
(Eq 1)



Bis(secondary amine) monomers that are useful in the present inventive method include, but are not limited to, N,N'-dimethylethylenediamine, piperazine, 2-methylpiperazine, 1,2-bis(N-ethylamino)ethylene, and 4,4'-trimethylenedipiperidine. Diacrylate monomers that are useful in the present invention include, but are not limited to, 1,4-butanediol diacrylate, 1,4-butanediol dimethacrylate, 1,2-ethanediol diacrylate, 1,6-hexanediol diacrylate, 1,5-pentanediol diacrylate, 2,5-hexanediol diacrylate, and 1,3-propanediol diacrylate. Each of the monomers is dissolved in an organic solvent (e.g., THF, $CH_2Cl_2$, MeOH, EtOH, $CHCl_3$, hexanes, toluene, benzene, $CCl_4$, glyme, diethyl ether, DMSO, DMF, etc.), preferably DMSO. The resulting solutions are combined, and the reaction mixture is heated to yield the desired polymer. In other embodiments, the reaction is performed without the use of a solvent thereby obviating the need for removing the solvent after the synthesis. The reaction mixture is then heated to a temperature ranging from 30° C. to 200° C., preferably 40° C. to 150° C., more preferably 50° C. to 100° C. In a particularly preferred embodiment, the reaction mixture is heated to approximately 40° C., 50° C., 60° C., 70° C., 80° C., or 90° C. In another particularly preferred embodiment, the reaction mixture is heated to approximately 75° C. In another embodiment, the reaction mixture is heated to approximately 100° C. The polymerization reaction may also be catalyzed. The reaction time may range from hours to days depending on the polymerization reaction and the reaction conditions. As would be appreciated by one of skill in the art, heating the reaction tends to speed up the rate of reaction requiring a shorter reaction time. As would be appreciated by one of skill in this art, the molecular weight of the synthesized polymer may be determined by the reaction conditions (e.g., temperature, starting materials, concentration, catalyst, solvent, time of reaction, etc.) used in the synthesis.

In another particularly preferred embodiment, the inventive polymers are prepared by the conjugate addition of a primary amine to a bis(acrylate ester). The use of primary amines rather than bis(secondary amines) allows for a much wider variety of commercially available starting materials. The reaction scheme using primary amines rather than secondary amines is shown below:

(Eq 2)



Primary amines useful in this method include, but are not limited to, methylamine, ethylamine, isopropylamine, aniline, substituted anilines, and ethanolamine. The bis(acrylate esters) useful in this method include, but are not limited to, 1,4-butanediol diacrylate, 1,4-butanediol dimethacrylate, 1,2-ethanediol diacrylate, 1,6-hexanediol diacrylate, 1,5-pentanediol diacrylate, 2,5-hexanediol diacrylate, and 1,3-propanediol diacrylate. Each of the monomers is dissolved in an organic solvent (e.g., THF, DMSO, DMF, $CH_2Cl_2$, MeOH, EtOH, $CHCl_3$, hexanes, $CCl_4$, glyme, diethyl ether, etc.). Organic solvents are preferred due to the susceptibility of polyesters to hydrolysis. Preferably the organic solvent used is relatively non-toxic in living systems. In certain embodiments, DMSO is used as the organic solvent because it is relatively non-toxic and is frequently used as a solvent in cell culture and in storing frozen stocks of cells. Other preferred solvents include those miscible with water such as DMSO, ethanol, and methanol. The resulting solutions of monomers which are preferably at a concentration between 0.01 M and 5 M, between approximately 0.1 M and 2 M, more preferably between 0.5 M and 2 M, and most preferably between 1.3 M and 1.8 M, are combined, and the reaction mixture is heated to yield the desired polymer. In certain embodiments, the reaction is run without solvent. Running the polymerization without solvent may decrease the amount of cyclization products resulting from intramolecular conjugate addition reactions. The polymerization may be run at a temperature ranging from 20° C. to 200° C., preferably from 40° C. to 100° C., more preferably from 50° C. to 75° C., even more preferably from 50° C. to 60° C. In a particularly preferred embodiment, the reaction mixture is maintained at 20° C. In another particularly preferred embodiment, the reaction mixture is heated to approximately 50° C. In some embodiments, the reaction mixture is heated to approximately 56° C. In yet another particularly preferred embodiment, the reaction mixture is heated to approximately 75° C. The reaction mixute may also be cooled to approximately 0° C. The polymerization reaction may also be catalyzed such as with an organometallic catalyste, acid, or base. In another preferred embodiment, one or more types of amine monomers and/or diacrylate monomers may be used in the polymerization reaction. For example, a combination of ethanolamine and ethylamine may be used to prepare a polymer more hydrophilic than one prepared using ethylamine alone, and also more hydrophobic than one prepared using ethanolamine alone.

In preparing the polymers of the present invention, the monomers in the reaction mixture may be combined in different ratio to effect molecular weight, yield, end-termination, etc. of the resulting polymer. The ratio of amine monomers to diacrylate monomers may range from 1.6 to 0.4, preferably from 1.4 to 0.6, more preferably from 1.2 to 0.8, even more preferably from 1.1 to 0.9. In certain embodiments, the ratio of amine monomers to diacrylate monomers is approximately 1.0. For example, combining the monomers at a ratio of 1:1 typically results in higher molecular weight polymers and higher overall yields. Also, providing an excess of amine monomers (i.e., amine-to-acrylate ratio>1) results in amine-terminated chains while providing an excess of acrylate monomer (i.e., amine-to-acrylate ration<1) results in acrylate-terminated chains.

The synthesized polymer may be purified by any technique known in the art including, but not limited to, precipitation, crystallization, chromatography, etc. In a particularly preferred embodiment, the polymer is purified through repeated precipitations in organic solvent (e.g., diethyl ether, hexane, etc.). In a particularly preferred embodiment, the polymer is isolated as a hydrochloride, phosphate, or acetate salt. The resulting polymer may also be used as is without further purification and isolation; thereby making it advantageous to use a solvent compatible with the assays to be used in assessing the polymers. For example, the polymers may be prepared in a non-toxic solvent such as DMSO, and the resulting solution of polymer may then be used in cell culture assays involving transfecting a nucleic acid into a cell. As would be appreciated by one of skill in this art, the molecular weight of the synthesized polymer and the extent of cross-linking may be determined by the reaction conditions (e.g., temperature, starting materials, concentration, order of addition, solvent, etc.) used in the synthesis (Odian Principles of Polymerization 3rd Ed., New York: John Wiley & Sons, 1991; Stevens Polymer Chemistry: An Introduction 2nd Ed., New York: Oxford University Press, 1990; each of which is incorporated herein by reference). The extent of cross-linking of the prepared polymer may be minimized to between 1-20%, preferably between 1-10%, more preferably between 1-5%, and most preferably less than 2%. As would be appreciated by those of skill in this art, amines or bis(acrylate ester)s with nucleophlic groups are more susceptible to cross-linking, and measures may need to be taken to reduce cross-linking such as lowering the temperature or changing the concetration of the starting materials in the reaction mixture. Acrylates and other moieties with unsaturation or halogens are also susceptible to radical polymerization which can lead to cross-linking. The extent of radical polymerization and cross-linking may be reduced by reducing the temperature of the reaction mixture or by other means known in the art.

In one embodiment, a library of different polymers is prepared in parallel. The synthesis of a library of polymers may be carried out using any of the teachings known in the art or described herein regarding the synthesis of polymers of the invention. In one embodiment, a different amine and/or bis (acrylate ester) at a particular amine-to-acrylate ratio is added to each vial in a set of vials used to prepare the library or to each well in a multi-well plate (e.g., 96-well plate). In one embodiment, the monomers are diluted to between 0.1 M and 5 M, more preferably 0.5 M to 2 M, and most preferably at approximately 1.6 M, in an organic solvent such as DMSO. The monomers may be combined in different ratio to effect molecular weight, yield, end-termination, etc. For example, combining the monomers at a ratio of 1:1 typically yields higher molecular weight polymers and higher overall yields. Providing an excess of amine monomer results in amine-terminated chains while providing an excess of acrylate monomer results in acrylate-terminated chains. In some embodiments, no solvent is used in the syntheis of the polymer. The array of vials or multi-well plate is incubated at a temperature and length of time sufficient to allow polymerization of the monomers to occur as described herein. In certain preferred embodiments, the time and temperature are chosen to effect near complete incorporation (e.g., 50% conversion, 75% conversion, 80% conversion, 90% conversion, 95% conversion, >95% conversion, 98% conversion, 99% conversion, or >99% conversion) of all the monomer into polymer. The polymerization reaction may be run at any temperatures ranging from 0° C. to 200° C. In one embodiment, the reaction mixtures are incubated at approximately 45° C. for approximately 5 days. In another embodiment, the reaction mixtures are incubated at approximately 56° C. for approximately 5 days. In aother embodiment, the reaction mixtures are incubated at approximately 100° C. for approximately 5 hours. The polymers may then be isolated and purified using techniques known in the art, or the resulting solutions of polymers may be used without further isolation or purification. In certain embodiments, over 1000 different polymers are prepared in parallel. In other embodiments, over 2000 different polymers are prepared in parallel. In still other embodiments, over 3000 different polymers are prepared in parallel. The polymers may then be screened using high-throughput techniques to identify polymers with a desired characteristic (e.g., solubility in water, solubility at different pH's, solubility in various organic solvents, ability to bind polynucleotides, ability to bind heparin, ability to bind small molecules, ability to form microparticles, ability to increase tranfection efficiency, etc.). The polymers of the invention may be screened or used after synthesis without further precipitation, purification, or isolation of the polymer. The use of a non-toxic solvent such as DMSO in the synthesis of the polymers allows for the easy handling and use of the polymers after the synthesis of the polymer. For instance, the solution of polymer in DMSO may be added to a cell culture or other living system without a toxic effect on the cells. In certain embodiments the polymers may be screened for properties or characteristics useful in gene therapy (e.g., ability to bind polynucleotides, increase in transfection efficiency, etc.). In other embodiments the polymers may be screened for properties or characteristics useful in the art of tissue engineering (e.g., ability to support tissue or cell growth, ability to promote cell attachment). In certain embodiments, the polymers are synthesized and assayed using semi-automated techniques and/or robotic fluid handling systems.

Polynucleotide Complexes

The ability of cationic compounds to interact with negatively charged polynucleotides through electrostatic interactions is well known. Cationic polymers such as poly(lysine) have been prepared and studied for their ability to complex polynucleotides. However, polymers studied to date have incorporated amines at the terminal ends of short, conformationally flexible side chains (e.g., poly(lysine)) or accessible amines on the surface of spherical or globular polyamines (e.g., PEI and PAMAM dendrimers). The interaction of the polymer with the polynucleotide is thought to at least partially prevent the degradation of the polynucleotide. By neutralizing the charge on the backbone of the polynucleotide, the neutral or slightly-positively-charged complex is also able to more easily pass through the hydrophobic membranes (e.g., cytoplasmic, lysosomal, endosomal, nuclear) of the cell. In a particularly preferred embodiment, the complex is slightly positively charged. In another particularly preferred embodiment, the complex has a postive ζ-potential, more preferably the ζ-potential is between +1 and +30. In certain embodiments, agents such as polyacrylic acid (pAA), poly aspartic acid, polyglutamic acid, or poly-maleic acid may be used to prevent the serum inhibition of the polynucleotide/polymer complexes in cultured cells in media with serum (Trubetskoy et al. "Recharging cationic DNA complexes with highly charged polyanions for in vitro and in vivo gene delivery" Gene Therapy 10:261-271, 2003; incorporated herein by reference).

The poly(β-amino esters) of the present invention possess tertiary amines in the backbone of the polymer. Although these amines are more hindered, they are available to interact with a polynucleotide. Polynucleotides or derivatives thereof are contacted with the inventive polymers under conditions suitable to form polynucleotide/polymer complexes. In certain embodiments, the ratio of nitrogen in the polymer (N) to phosphate in the polynucleotide is 10:1, 15:1, 20:1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110: 1, or 120:1. In certain embodiments, the polymer-to-DNA (w/w) ratio is 10: 1, 15:1, 20: 1, 25:1, 30:1, 35:1, 40:1, 45:1, 50:1, 55:1, 60:1, 65:1, 70:1, 75:1, 80:1, 85:1, 90:1, 95:1, 100:1, 110:1, 120:1, 130:1, 140:1, 150:1, or 200:1. The polymer is preferably at least partially protonated so as to form a complex with the negatively charged polynucleotide. In a preferred embodiment, the polynucleotide/polymer complexes form nanoparticles that are useful in the delivery of polynucleotides to cells. In a particularly preferred embodiment, the diameter of the nanoparticles ranges from 50-500 nm, more preferably the diameter of the nanoparticles ranges from 50-200 nm, and most preferably from 90-150 nm. The nanoparticles may be associated with a targeting agent as described below.

In certain embodiments, other agents may be added to the polynucleotide:poly(betamino ester) complexes. In certain embodiments, a co-complexing agent is used. Co-complexing agents are known to bind polynucleotides and/or increase transfection efficiency. Co-complexing agents usually have a high nitrogen density. Polylysine (PLL) and polyethylenimine (PEI) are two examples of polymeric co-complexing agents. PLL has a molecular weight to nitrogen atom ratio of 65, and PEI has a molecular weight to nitrogen atom ratio of

43. Any polymer with a molecular weight to nitrogen atom ratio in the range of 10-100, preferably 25-75, more preferably 40-70, may be useful as a co-complexing agent. The inclusion of a co-complexing agent in a complex may allow one to reduce the amount of poly(beta-amino ester) in the complex. This becomes particularly important if the poly (beta-amino ester) is cytotoxic at higher concentrations. In the resulting complexes with co-complexing agents, the co-complexing agent to polynucleotide (w/w) ratio may range from 0 to 2.0, preferably from 0.1 to 1.2, more preferably from 0.1 to 0.6, and even more preferably from 0.1 to 0.4.

The transfection properties of various complexes of the invention may be determined by in vitro transfection studies (e.g., GFP transfection in cultured cells) or in animal models. In certain embodiments, the complex used for transfection is optimized for a particular cell type, polynucleotide to be delivered, poly(beta-amino ester), co-complexing agent (if one is used), disease process, method of administration (e.g., inhalation, oral, parenteral, IV, intrathecal, etc.), dosage regimen, etc.

Polynucleotide

The polynucleotide to be complexed or encapsulated by the inventive polymers may be any nucleic acid including but not limited to RNA and DNA. The polynucleotides may be of any size or sequence, and they may be single- or double-stranded. In certain preferred embodiments, the polynucleotide is greater than 100 base pairs long. In certain other preferred embodiments, the polynucleotide is greater than 1000 base pairs long and may be greater than 10,000 base pairs long. The polynucleotide is preferably purified and substantially pure. Preferably, the polynucleotide is greater than 50% pure, more preferably greater than 75% pure, and most preferably greater than 95% pure. The polynucleotide may be provided by any means known in the art. In certain preferred embodiments, the polynucleotide has been engineered using recombinant techniques (for a more detailed description of these techniques, please see Ausubel et al. Current Protocols in Molecular Biology (John Wiley & Sons, Inc., New York, 1999); Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press: 1989); each of which is incorporated herein by reference). The polynucleotide may also be obtained from natural sources and purified from contaminating components found normally in nature. The polynucleotide may also be chemically synthesized in a laboratory. In a preferred embodiment, the polynucleotide is synthesized using standard solid phase chemistry.

The polynucleotide may be modified by chemical or biological means. In certain preferred embodiments, these modifications lead to increased stability of the polynucleotide. Modifications include methylation, phosphorylation, end-capping, etc.

Derivatives of polynucleotides may also be used in the present invention. These derivatives include modifications in the bases, sugars, and/or phosphate linkages of the polynucleotide. Modified bases include, but are not limited to, those found in the following nucleoside analogs: 2-aminoadenosine, 2-thiothymidine, inosine, pyrrolo-pyrimidine, 3-methyl adenosine, 5-methylcytidine, C5-bromouridine, C5-fluorouridine, C5-iodouridine, C5-propynyl-uridine, C5-propynyl-cytidine, C5-methylcytidine, 7-deazaadenosine, 7-deazaguanosine, 8-oxoadenosine, 8-oxoguanosine, O(6)-methylguanine, and 2-thiocytidine. Modified sugars include, but are not limited to, 2'-fluororibose, ribose, 2'-deoxyribose, 3'-azido-2',3'-dideoxyribose, 2',3'-dideoxyribose, arabinose (the 2'-epimer of ribose), acyclic sugars, and hexoses. The nucleosides may be strung together by linkages other than the phosphodiester linkage found in naturally occurring DNA and RNA. Modified linkages include, but are not limited to, phosphorothioate and 5'-N-phosphoramidite linkages. Combinations of the various modifications may be used in a single polynucleotide. These modified polynucleotides may be provided by any means known in the art; however, as will be appreciated by those of skill in this art, the modified polynucleotides are preferably prepared using synthetic chemistry in vitro.

The polynucleotides to be delivered may be in any form. For example, the polynucleotide may be a circular plasmid, a linearized plasmid, a cosmid, a viral genome, a modified viral genome, an artificial chromosome, etc.

The polynucleotide may be of any sequence. In certain preferred embodiments, the polynucleotide encodes a protein or peptide. The encoded proteins may be enzymes, structural proteins, receptors, soluble receptors, ion channels, pharmaceutically active proteins, cytokines, interleukins, antibodies, antibody fragments, antigens, coagulation factors, albumin, growth factors, hormones, insulin, etc. The polynucleotide may also comprise regulatory regions to control the expression of a gene. These regulatory regions may include, but are not limited to, promoters, enhancer elements, repressor elements, TATA box, ribosomal binding sites, stop site for transcription, etc. In other particularly preferred embodiments, the polynucleotide is not intended to encode a protein. For example, the polynucleotide may be used to fix an error in the genome of the cell being transfected.

The polynucleotide may also be provided as an antisense agent or RNA interference (RNAi) (Fire et al. Nature 391: 806-811, 1998; incorporated herein by reference). Antisense therapy is meant to include, e.g., administration or in situ provision of single- or double-stranded oligonucleotides or their derivatives which specifically hybridize, e.g., bind, under cellular conditions, with cellular mRNA and/or genomic DNA, or mutants thereof, so as to inhibit expression of the encoded protein, e.g., by inhibiting transcription and/or translation (Crooke "Molecular mechanisms of action of antisense drugs" Biochim. Biophys. Acta 1489(1):31-44, 1999; Crooke "Evaluating the mechanism of action of antiproliferative antisense drugs" Antisense Nucleic Acid Drug Dev. 10(2):123-126, discussion 127, 2000; Methods in Enzymology volumes 313-314, 1999; each of which is incorporated herein by reference). The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major groove of the double helix (i.e., triple helix formation) (Chan et al. J. Mol. Med. 75(4):267-282, 1997; incorporated herein by reference).

In a particularly preferred embodiment, the polynucleotide to be delivered comprises a sequence encoding an antigenic peptide or protein. Nanoparticles containing these polynucleotides can be delivered to an individual to induce an immunologic response sufficient to decrease the chance of a subsequent infection and/or lessen the symptoms associated with such an infection. The polynucleotide of these vaccines may be combined with interleukins, interferon, cytokines, and adjuvants such as cholera toxin, alum, Freund's adjuvant, etc. A large number of adjuvant compounds are known; a useful compendium of many such compounds is prepared by the National Institutes of Health and can be found on the World Wide Web (http:/www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf, incorporated herein by reference; see also Allison Dev. Biol. Stand. 92:3-11, 1998; Unkeless et al. Annu. Rev. Immunol. 6:251-281, 1998; and Phillips et al. Vaccine 10:151-158,1992, each of which is incorporated herein by reference).

The antigenic protein or peptides encoded by the polynucleotide may be derived from such bacterial organisms as Streptococccus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium diphtheriae, Listeria monocytogenes, Bacillus anthracis, Clostridium tetani, Clostridium botulinum, Clostridium perfringens, Neisseria meningitidis, Neisseria gonorrhoeae, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhi, Haemophilus parainfluenzae, Bordetella pertussis, Francisella tularensis, Yersinia pestis, Vibrio cholerae, Legionella pneumophila, Mycobacterium tuberculosis, Mycobacterium leprae, Treponema pallidum, Leptospirosis interrogans, Borrelia burgdorferi, Camphylobacter jejuni, and the like; from such viruses as smallpox, influenza A and B, respiratory syncytial virus, parainfluenza, measles, HIV, varicella-zoster, herpes simplex 1 and 2, cytomegalovirus, Epstein-Barr virus, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rabies, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, hepatitis A, B, C, D, and E virus, and the like; and from such fungal, protozoan, and parasitic organisms such as Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasma pneumoniae, Chlamydial psittaci, Chlamydial trachomatis, Plasmodium falciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis, Schistosoma mansoni, and the like.

Microparticles

The poly(β-amino esters) of the present invention may also be used to form drug delivery devices. The inventive polymers may be used to encapsulate agents including polynucleotides, small molecules, proteins, peptides, metals, organometallic compounds, etc. The inventive polymers have several properties that make them particularly suitable in the preparation of drug delivery devices. These include 1) the ability of the polymer to complex and "protect" labile agents; 2) the ability to buffer the pH in the endosome; 3) the ability to act as a "proton sponge" and cause endosomolysis; and 4) the ability to neutralize the charge on negatively charged agents. In a preferred embodiment, the polymers are used to form microparticles containing the agent to be delivered. In a particularly preferred embodiment, the diameter of the microparticles ranges from between 500 nm to 50 micrometers, more preferably from 1 micrometer to 20 micrometers, and most preferably from 1 micrometer to 10 micrometers. In another particularly preferred embodiment, the microparticles range from 1-5 micrometers. The encapsulating inventive polymer may be combined with other polymers (e.g., PEG, PLGA) to form the microspheres.

Methods of Preparing Microparticles

The inventive microparticles may be prepared using any method known in this art. These include, but are not limited to, spray drying, single and double emulsion solvent evaporation, solvent extraction, phase separation, simple and complex coacervation, and other methods well known to those of ordinary skill in the art. Particularly preferred methods of preparing the particles are the double emulsion process and spray drying. The conditions used in preparing the microparticles may be altered to yield particles of a desired size or property (e.g., hydrophobicity, hydrophilicity, external morphology, "stickiness", shape, etc.). The method of preparing the particle and the conditions (e.g., solvent, temperature, concentration, air flow rate, etc.) used may also depend on the agent being encapsulated and/or the composition of the polymer matrix.

Methods developed for making microparticles for delivery of encapsulated agents are described in the literature (for example, please see Doubrow, M., Ed., "Microcapsules and Nanoparticles in Medicine and Pharmacy," CRC Press, Boca Raton, 1992; Mathiowitz and Langer, J Controlled Release 5:13-22, 1987; Mathiowitz et al. Reactive Polymers 6:275-283, 1987; Mathiowitz et al. J. Appl. Polymer Sci. 35:755-774, 1988; each of which is incorporated herein by reference).

If the particles prepared by any of the above methods have a size range outside of the desired range, the particles can be sized, for example, using a sieve.

Agent

The agents to be delivered by the system of the present invention may be therapeutic, diagnostic, or prophylactic agents. Any chemical compound to be administered to an individual may be delivered using the inventive microparticles. The agent may be a small molecule, organometallic compound, nucleic acid, protein, peptide, polynucleotide, metal, an isotopically labeled chemical compound, drug, vaccine, immunological agent, etc.

In a preferred embodiment, the agents are organic compounds with pharmaceutical activity. In another embodiment of the invention, the agent is a clinically used drug. In a particularly preferred embodiment, the drug is an antibiotic, anti-viral agent, anesthetic, steroidal agent, anti-inflammatory agent, anti-neoplastic agent, antigen, vaccine, antibody, decongestant, antihypertensive, sedative, birth control agent, progestational agent, anti-cholinergic, analgesic, anti-depressant, anti-psychotic, β-adrenergic blocking agent, diuretic, cardiovascular active agent, vasoactive agent, non-steroidal anti-inflammatory agent, nutritional agent, etc.

In a preferred embodiment of the present invention, the agent to be delivered may be a mixture of agents. For example, a local anesthetic may be delivered in combination with an anti-inflammatory agent such as a steroid. Local anesthetics may also be administered with vasoactive agents such as epinephrine. To give another example, an antibiotic may be combined with an inhibitor of the enzyme commonly produced by bacteria to inactivate the antibiotic (e.g., penicillin and clavulanic acid).

Diagnostic agents include gases; metals; commercially available imaging agents used in positron emissions tomography (PET), computer assisted tomography (CAT), single photon emission computerized tomography, x-ray, fluoroscopy, and magnetic resonance imaging (MRI); and contrast agents. Examples of suitable materials for use as contrast agents in MRI include gadolinium chelates, as well as iron, magnesium, manganese, copper, and chromium. Examples of materials useful for CAT and x-ray imaging include iodine-based materials.

Prophylactic agents include, but are not limited to, antibiotics, nutritional supplements, and vaccines. Vaccines may comprise isolated proteins or peptides, inactivated organisms and viruses, dead organisms and viruses, genetically altered organisms or viruses, and cell extracts. Prophylactic agents may be combined with interleukins, interferon, cytokines, and adjuvants such as cholera toxin, alum, Freund's adjuvant, etc. Prophylactic agents include antigens of such bacterial organisms as Streptococccus pneumoniae, Haemophilus influenzae, Staphylococcus aureus, Streptococcus pyrogenes, Corynebacterium diphtheriae, Listeria monocytogenes, Bacillus anthracis, Clostridium tetani, Clostridium botulinum, Clostridium perfringens, Neisseria meningitidis, Neisseria gonorrhoeae, Streptococcus mutans, Pseudomonas aeruginosa, Salmonella typhi, Haemophilus parainfluenzae, Bordetella pertussis, Francisella tularensis, Yersinia pestis, Vibrio cholerae, Legionella pneumophila, Mycobacterium tuberculosis, Mycobacterium leprae, Treponemapallidum, Leptospirosis interrogans, Borrelia burgdorferi, Camphylobacter jejuni, and the like; antigens of such viruses as smallpox, influenza A and B, respiratory syncytial virus, parainfluenza, measles, HIV, varicella-zoster, herpes simplex 1 and 2, cytomegalovirus, Epstein-Barr virus, rotavirus, rhinovirus, adenovirus, papillomavirus, poliovirus, mumps, rabies, rubella, coxsackieviruses, equine encephalitis, Japanese encephalitis, yellow fever, Rift Valley fever, hepatitis A, B, C, D, and E virus, and the like; antigens of fungal, protozoan, and parasitic organisms such as Cryptococcus neoformans, Histoplasma capsulatum, Candida albicans, Candida tropicalis, Nocardia asteroides, Rickettsia ricketsii, Rickettsia typhi, Mycoplasmapneumoniae, Chlamydialpsittaci, Chlamydial trachomatis, Plasmodiumfalciparum, Trypanosoma brucei, Entamoeba histolytica, Toxoplasma gondii, Trichomonas vaginalis, Schistosoma mansoni, and the like. These antigens may be in the form of whole killed organisms, peptides, proteins, glycoproteins, carbohydrates, or combinations thereof.

Targeting Agents

The inventive micro- and nanoparticles may be modified to include targeting agents since it is often desirable to target a particular cell, collection of cells, or tissue. A variety of targeting agents that direct pharmaceutical compositions to particular cells are known in the art (see, for example, Cotten et al. Methods Enzym. 217:618, 1993; incorporated herein by reference). The targeting agents may be included throughout the particle or may be only on the surface. The targeting agent may be a protein, peptide, carbohydrate, glycoprotein, lipid, small molecule, etc. The targeting agent may be used to target specific cells or tissues or may be used to promote endocytosis or phagocytosis of the particle. Examples of targeting agents include, but are not limited to, antibodies, fragments of antibodies, low-density lipoproteins (LDLs), transferrin, asialycoproteins, gp120 envelope protein of the human immunodeficiency virus (HIV), carbohydrates, receptor ligands, sialic acid, etc. If the targeting agent is included throughout the particle, the targeting agent may be included in the mixture that is used to form the particles. If the targeting agent is only on the surface, the targeting agent may be associated with (i.e., by covalent, hydrophobic, hydrogen boding, van der Waals, or other interactions) the formed particles using standard chemical techniques.

Pharmaceutical Compositions

Once the microparticles or nanoparticles (polymer complexed with polynucleotide) have been prepared, they may be combined with one or more pharmaceutical excipients to form a pharmaceutical composition that is suitable to administer to animals including humans. As would be appreciated by one of skill in this art, the excipients may be chosen based on the route of administration as described below, the agent being delivered, time course of delivery of the agent, etc.

Pharmaceutical compositions of the present invention and for use in accordance with the present invention may include a pharmaceutically acceptable excipient or carrier. As used herein, the term "pharmaceutically acceptable carrier" means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are sugars such as lactose, glucose, and sucrose; starches such as corn starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil; safflower oil; sesame oil; olive oil; corn oil and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; detergents such as Tween 80; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; and phosphate buffer solutions, as well as other non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, releasing agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the composition, according to the judgment of the formulator. The pharmaceutical compositions of this invention can be administered to humans and/or to animals, orally, rectally, parenterally, intracistemally, intravaginally, intranasally, intraperitoneally, topically (as by powders, creams, ointments, or drops), bucally, or as an oral or nasal spray.

Liquid dosage forms for oral administration include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups, and elixirs. In addition to the active ingredients (i.e., microparticles, nanoparticles, polynucleotide/polymer complexes), the liquid dosage forms may contain inert diluents commonly used in the art such as, for example, water or other solvents, solubilizing agents and emulsifiers such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, dimethylformamide, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor, and sesame oils), glycerol, tetrahydrofurfuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof. Besides inert diluents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, and perfuming agents.

Injectable preparations, for example, sterile injectable aqueous or oleaginous suspensions may be formulated according to the known art using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation may also be a sterile injectable solution, suspension, or emulsion in a nontoxic parenterally acceptable diluent or solvent, for example, as a solution in 1,3-butanediol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, U.S.P. and isotonic sodium chloride solution. In addition, sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic mono- or diglycerides. In addition, fatty acids such as oleic acid are used in the preparation of injectables. In a particularly preferred embodiment, the particles are suspended in a carrier fluid comprising 1% (w/v) sodium carboxymethyl cellulose and 0.1% (v/v) Tween 80.

The injectable formulations can be sterilized, for example, by filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable medium prior to use.

Compositions for rectal or vaginal administration are preferably suppositories which can be prepared by mixing the particles with suitable non-irritating excipients or carriers such as cocoa butter, polyethylene glycol, or a suppository wax which are solid at ambient temperature but liquid at body temperature and therefore melt in the rectum or vaginal cavity and release the microparticles.

Solid dosage forms for oral administration include capsules, tablets, pills, powders, and granules. In such solid dosage forms, the particles are mixed with at least one inert, pharmaceutically acceptable excipient or carrier such as sodium citrate or dicalcium phosphate and/or a) fillers or extenders such as starches, lactose, sucrose, glucose, mannitol, and silicic acid, b) binders such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinylpyrrolidinone, sucrose, and acacia, c) humectants such as glycerol, d) disintegrating agents such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate, e) solution retarding agents such as paraffin, f) absorption accelerators such as quaternary ammonium compounds, g) wetting agents such as, for example, cetyl alcohol and glycerol monostearate, h) absorbents such as kaolin and bentonite clay, and i) lubricants such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof. In the case of capsules, tablets, and pills, the dosage form may also comprise buffering agents.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

The solid dosage forms of tablets, dragees, capsules, pills, and granules can be prepared with coatings and shells such as enteric coatings and other coatings well known in the pharmaceutical formulating art. They may optionally contain opacifying agents and can also be of a composition that they release the active ingredient(s) only, or preferentially, in a certain part of the intestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes.

Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugar as well as high molecular weight polyethylene glycols and the like.

Dosage forms for topical or transdermal administration of an inventive pharmaceutical composition include ointments, pastes, creams, lotions, gels, powders, solutions, sprays, inhalants, or patches. The particles are admixed under sterile conditions with a pharmaceutically acceptable carrier and any needed preservatives or buffers as may be required. Ophthalmic formulation, ear drops, and eye drops are also contemplated as being within the scope of this invention.

The ointments, pastes, creams, and gels may contain, in addition to the particles of this invention, excipients such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc, and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to the particles of this invention, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates, and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants such as chlorofluorohydrocarbons.

Transdermal patches have the added advantage of providing controlled delivery of a compound to the body. Such dosage forms can be made by dissolving or dispensing the microparticles or nanoparticles in a proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate can be controlled by either providing a rate controlling membrane or by dispersing the particles in a polymer matrix or gel.

These and other aspects of the present invention will be further appreciated upon consideration of the following Examples, which are intended to illustrate certain particular embodiments of the invention but are not intended to limit its scope, as defined by the claims.

## EXAMPLES

### Example 1

### Degradable Poly(β-Amino Esters): Synthesis, Characterization, and Self-Assembly with Plasmid DNA

Experimental Section

General Considerations. All manipulations involving live cells or sterile materials were performed in a laminar flow using standard sterile technique. $^1H$ NMR (300.100 MHz) and $^{13}C$ NMR (75.467 MHz) spectra were recorded on a Varian Mercury spectrometer. All chemical shift values are given in ppm and are referenced with respect to residual proton or carbon signal from solvent. Organic phase gel permeation chromatography (GPC) was performed using a Hewlett Packard 1100 Series isocratic pump, a Rheodyneu Model 7125 injector with a 100-μL injection loop, and two PL-Gel mixed-D columns in series (5 μm, 300×7.5 mm, Polymer Laboratories, Amherst, Mass.). THF/0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, Calif.) and processed using the TriSEC GPC software package (Viscotek Corporation, Houston, Tex.). The molecular weights and polydispersities of the polymers are reported relative to monodispersed polystyrene standards. Aqueous phase GPC was performed by American Polymer Standards (Mentor, Ohio) using Ultrahydrogel L and 120A columns in series (Waters Corporation, Milford, Mass.). Water (1% acetic acid, 0.3 M NaCl) was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using a Knauer differential refractometer and processed using an IBM/PC GPC-PRO3.13 software package (Viscotek Corporation, Houston, Tex.). The molecular weights and polydispersities of the polymers are reported relative to poly(2-vinylpyridine) standards. For cytotoxicity assays, absorbance was measured using a Dynatech Laboratories MR5000 microplate reader at 560 nmn.

Materials. N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine were purchased from Aldrich Chemical Company (Milwaukee, Wis.). 1,4-butanediol diacrylate was purchased from Alfa Aesar Organics (Ward Hill, Mass.). (3-[4,5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St. Louis, Mo.). Plasmid DNA (pCMV-Luc) was produced in E. coli (DH5α, a kind gift from Zycos, Inc., Cambridge, Mass.), isolated with a Qiagen kit, and purified by ethanol precipitation. NIH 3T3 cells were purchased from American Type Culture Collection (Manassas, Va.) and grown at 37° C., 5% $CO_2$ in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL. All other materials and solvents were used as received without further purification.

General Polymerization Procedure. In a typical experiment, 1,4-butanediol diacrylate (0.750 g, 0.714 mL, 3.78 mmol) and diamine (3.78 mmol) were weighed into two separate vials and dissolved in THF (5 mL). The solution containing the diamine was added to the diacrylate solution via pipette. A Teflon-coated stirbar was added, the vial was sealed with a Teflon-lined screw-cap, and the reaction was heated at 50° C. After 48 hours, the reaction was cooled to room temperature and dripped slowly into vigorously stirring diethyl ether or hexanes. Polymer was collected and dried under vacuum prior to analysis.

Synthesis of Polymer 1. Polymer 1 was prepared according to the general procedure outlined above. $^1$H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4H), 2.75 (br t, J=7.05 Hz, 4H), 2.53 (br s, 4H), 2.50 (br t, (obsc), J=7.20 Hz, 4H), 2.28 (br s, 6H), 1.71, (br m, 4H). $^{13}$C NMR δ ($CDCl_3$, 75.47 MHz) 172.55, 64.14, 55.31, 53.39, 42.47, 32.54, 25.53.

Synthesis of Polymer 2. Polymer 2 was prepared according to the general procedure outlined above. $^1$H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4H), 2.74 (br t, J=7.35, 4H), 2.56 (br m, 12H), 1.71 (br t, 4H). $^{13}$C NMR δ ($CDCl_3$, 75.47 MHz) 172.24, 64.19, 53.55, 52.75, 32.27, 25.52.

Synthesis of Polymer 3. Polymer 3 was prepared according to the general procedure outlined above. $^1$H NMR δ ($CDCl_3$, 300 MHz) 4.11 (br t, 4H), 3.00 (br m, 4H), 2.79 (br m, 4H), 2.65 (br m, 4H), 2.11 (br m, 4H), 1.70 (br m, 8H), 1.25 (br m, 12H). $^{13}$C NMR δ ($CDCl_3$, 75.47 MHz) 172.37, 64.13, 53.89 (br), 36.74, 35.58, 32.11 (br), 25.45, 24.05.

Polymer Degradation Studies. The hydrochloride salts of polymers 1-3 were dissolved in acetate buffer (1 M, pH=5.1) or HEPES buffer (1 M, pH=7.4) at a concentration of 5 mg/mL (the use of millimolar concentrations of buffer resulted in substantial reduction of pH during degradation due to the production of acidic degradation products). Samples were incubated at 37° C. on a mechanical rotator, and aliquots (1 mL) were removed at appropriate time intervals. Aliquots were frozen immediately in liquid nitrogen and lyophilized. Polymer was extracted from dried buffer salts using THF/0.1 M piperidine (1 mL), and samples were analyzed directly by GPC.

Formation of DNA/Polymer Complexes and Agarose Gel Retardation Assays.

DNA/polymer complexes were formed by adding 50 μL of a plasmid DNA solution (pCMV Luc, 2 μg/50 μL in water) to a gently vortexing solution of the hydrochloride salt of polymers 1-3 (50 μL in 25 mM MES, pH=6.0, concentrations adjusted to yield desired DNA/polymer weight ratios). The samples were incubated at room temperature for 30 minutes, after which 20%L was run on a 1% agarose gel (HEPES, 20 mM, pH=7.2, 65V, 30 min). Samples were loaded on the gel with a loading buffer consisting of 10% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) in HEPES (25 mM, pH=7.2). Bromphenol blue was not included as a visual indicator in the loading buffer, since this charged dye appeared to interfere with the complexation of polymer and DNA. DNA bands were visualized under UV illumination by ethidium bromide staining.

Quasi-Elastic Laser Light Scattering (QELS) and Measurement of ζ-potentials. QELS experiments and ζ-potential measurements were made using a ZetaPALS dynamic light scattering detector (Brookhaven Instruments Corporation, Holtsville, N.Y., 15 mW laser, incident beam=676 nm). DNA/polymer complexes were formed as described above for agarose gel retardation assays. Samples were diluted with 900 μL of HEPES (20 mM, pH=7.0), added to a gently vortexing sample of DNA/polymer complex (total volume=1 mL, pH=7.0). Average particle sizes and ζ-potentials were determined at 25° C. Correlation functions were collected at a scattering angle of 90°, and particle sizes were calculated using the MAS option of BIC's particle sizing software (ver. 2.30) using the viscosity and refractive index of pure water at 25° C. Particle sizes are expressed as effective diameters assuming a lognormal distribution. Average electrophoretic mobilities were measured at 25° C. using BIC PALS zeta potential analysis software and zeta potentials were calculated using the Smoluchowsky model for aqueous suspensions. Three measurements were made on each sample, and the results are reported as average diameters and zeta potentials.

Cytotoxicity Assays. Immortalized NIH 3T3 cells were grown in 96-well plates at an initial seeding density of 10,000 cells/well in 200 μL growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 hours, after which the growth medium was removed and replaced with 180 μL of serum-free medium. Appropriate amounts of polymer were added in 20 μL aliquots. Samples were incubated at 37° C. for 5 hours, and the metabolic activity of each well was determined using a MTT/thiazolyl blue assay: to each well was added 25 μL of a 5 mg/mL solution of MTT stock solution in sterile PBS buffer. The samples were incubated at 37° C. for 2 hours, and 100 μL of extraction buffer (20% w/v SDS in DMF/water (1:1), pH=4.7) was added to each well. Samples were incubated at 37° C. for 24 hours. Optical absorbance was measured at 560 nm with a microplate reader and expressed as a percent relative to control cells.

Results and Discussion

Polymer Synthesis and Characterization

The synthesis of linear poly(amido amines) containing tertiary amines in their backbones was reported by Ferruti et al. in 1970 via the addition of bifunctional amines to bisacrylamides (Anderson Nature 392(Suppl.):25-30, 1996; Friedman Nature Med. 2:144-147, 1996; Crystal Science 270:404-410, 1995; Mulligan Science 260:926-932, 1993; each of which is incorporated herein by reference). Linear poly(amido amines) were initially investigated as heparin and ion complexing biomaterials (Ferruti et al. Advances in Polymer Science 58:55-92, 1984; Ferruti et al. Biomaterials 15:1235-1241, 1994; Ferruti et al. Macromol. Chem. Phys. 200:1644-1654, 1999; Ferruti et al. Biomaterials 15:1235-1241, 1994; each of which is incorporated herein by reference). Dendritic poly(amido amines) (PAMAMs) have seen increasing use in gene transfer applications due to their ability to complex DNA (Kukowska-Latallo et al. Proc. Natl. Acad. Sci. USA 93:4897-4902, 1996; Tang et al. Bioconjugate Chem. 7:703-714, 1996; Haensler et al. Bioconjugate Chem. 4:372-379, 1993; each of which is incorporated herein by reference), and a recent report describes the application of a family of linear poly(amido amines) to cell transfection and cytotoxicity studies (Hill et al. Biochim. Biophys. Acta 1427:161-174, 1999; incorporated herein by reference). In contrast, analogous poly (ester amines) formed from the Michael-type addition of bifunctional amines to diacrylate esters have received less attention (Danusso et al. Polymer 11:88-113, 1970; Ferruti et al. Polymer 26:1336, 1985; Ferruti et al. Advances in Polymer Science 58:55-92, 1984; Ferruti et al. Biomaterials 15:1235-1241, 1994; Ferruti et al. Macromol. Chem. Phys. 200:1644-1654, 1999; Ferruti et al. Biomaterials 15:1235-1241, 1994; Kargina et al. Vysokomol. Soedin. Seriya A 28:1139-1144, 1986; Rao et al. J. Bioactive and Compatible Polymers 14:54-63, 1999; each of which is incorporated herein by reference).

The poly(amino ester) approach presents a particularly attractive basis for the development of new polymeric transfection vectors for several reasons: 1) the polymers contain the requisite amines and readily degradable linkages, 2) multiple analogs could potentially be synthesized directly from commercially available starting materials, and 3) if the resulting polymers were useful as DNA condensing agents, future generations of polymer could easily be engineered to possess amine $pK_a$ values spanning the range of physiologically relevant pH. This last point was particularly intriguing, because the buffering capacity of polyamines has recently been implicated as a factor influencing the escape of DNA from cell endosomes following endocytosis (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; Haensler et al. Bioconjugate Chem. 4:372-379, 1993; Behr Chimia 51:34-36, 1997;

Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Felgner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference). While the complexation of DNA with a cationic polymer is required to compact and protect DNA during early events in the transfection process, DNA and polymer must ultimately decomplex in the nucleus to allow efficient transcription (Luo et al. Nat. Biotechnol. 18:33-37, 2000; incorporated herein by reference). In view of this requirement, degradable polycations could play an important role in "vector unpackaging" events in the nucleus (Luo et al. Nat. Biotechnol. 18:33-37, 2000; Schaffer et al. Biotechnol. Bioeng. 67:598-606, 2000; Kabanov Pharm. Sci. Technol. Today 2:365-372, 1999; each of which is incorporated herein by reference). Finally, we hypothesized that polymers of this general structure, and the β-amino acid derivatives into which they would presumably degrade, would be significantly less toxic than poly(lysine) and PEI. As outlined above, degradable polycations (Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; each of which is incorporated herein by reference) and linear polymers containing relatively hindered amines located close to the polymer backbone (Gonzalez et al. Bioconjugate Chem. 10:1068-1074, 1999; incorporated herein by reference) are less toxic than poly (lysine) and PEI.

The synthesis of polymers 1-3 via the addition of the bis (secondary amines), N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine, to 1,4-butanediol diacrylate was investigated (Danusso et al. Polymer 11:88-113, 1970; Kargina et al. Vysokomol. Soedin. Seriya A 28:1139-1144, 1986; each of which is incorporated herein by reference). The polymerization of these monomers proceeded in THF and $CH_2Cl_2$ at 50° C. to yield the corresponding polymers in up to 86% yields (Table 1). Polymers were purified through repeated precipitation into diethyl ether or hexane. Polymer 1 was isolated as a clear viscous liquid; polymers 2 and 3 were obtained as white solids after drying under high vacuum. Alternatively, polymers 1-3 could be isolated as solid hydrochloride salts upon addition of diethyl ether/HCl to a solution of polymer in THF or $CH_2Cl_2$. All three polymers were soluble in organic solvents such as THF, $CH_2Cl_2$, $CHCl_3$, and MeOH and were also soluble in water at reduced pH. Polymer 1 and the hydrochloride salts of polymers 1-3 were freely soluble in water.

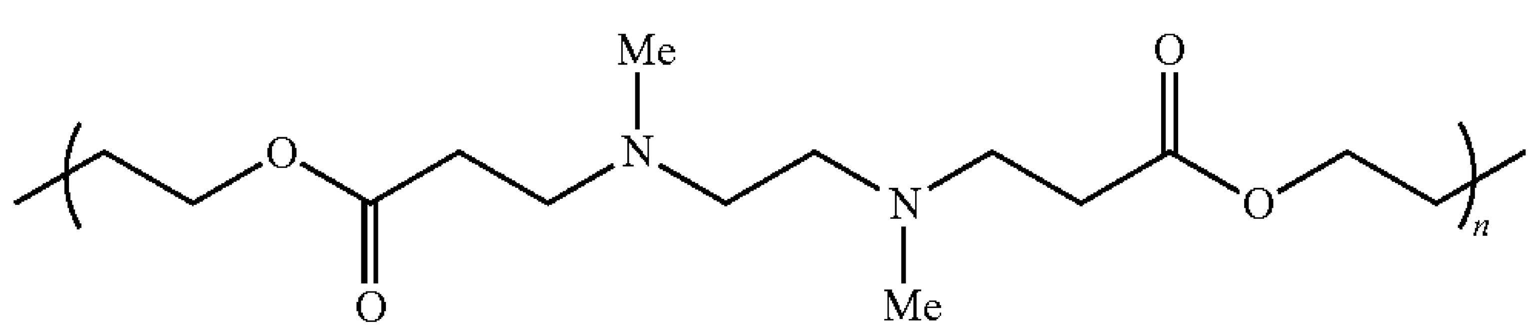
The molecular weights of polymers 1-3 and their corresponding hydrochloride salts were determined by both organic and aqueous phase gel permeation chromatography (GPC). Polymer molecular weights ($M_n$) ranged from up to 5,800 for polymer 1 to up to 32,000 for polymer 3, relative to polystyrene standards. Molecular weight distributions for these polymers were monomodal with polydispersity indices (PDIs) ranging from 1.55 to 2.55. Representative molecular weight data are presented in Table 1. While the synthesis of linear poly(amido amines) is generally performed using alcohols or water as solvents (Danusso et al. Polymer 11:88-113, 1970; Ferruti et al. Polymer 26:1336, 1985; Ferruti et al. Advances in Polymer Science 58:55-92, 1984; Ferruti et al. Biomaterials 15:1235-1241, 1994; Ferruti et al. Macromol. Chem. Phys. 200:1644-1654, 1999; Ferruti et al. Biomaterials 15:1235-1241, 1994; each of which is incorporated herein by reference), anhydrous THF and $CH_2Cl_2$ were employed in the synthesis of poly(β-amino esters) to minimize hydrolysis reactions during the synthesis. The yields and molecular weights of polymers synthesized employing $CH_2Cl_2$ as solvent were generally higher than those of polymers synthesized in THF (Table 1) (Polymer 1 could not by synthesized in $CH_2Cl_2$. The color of the reaction solution progressed from colorless to an intense pink color almost immediately after the introduction of a solution of N,N'-dimethylethylenediamine in $CH_2Cl_2$ to a solution of 1,4-butanediol diacrylate in $CH_2Cl_2$ (see Experimental Section above). The color progressed to light orange over the course of the reaction, and an orange polymer was isolated after precipitation into hexane. The isolated polymer was insoluble in $CH_2Cl_2$, THF, and water at reduced pH and was not structurally characterized. This problem was not encountered for the analogous reaction in THF.).

TABLE 1

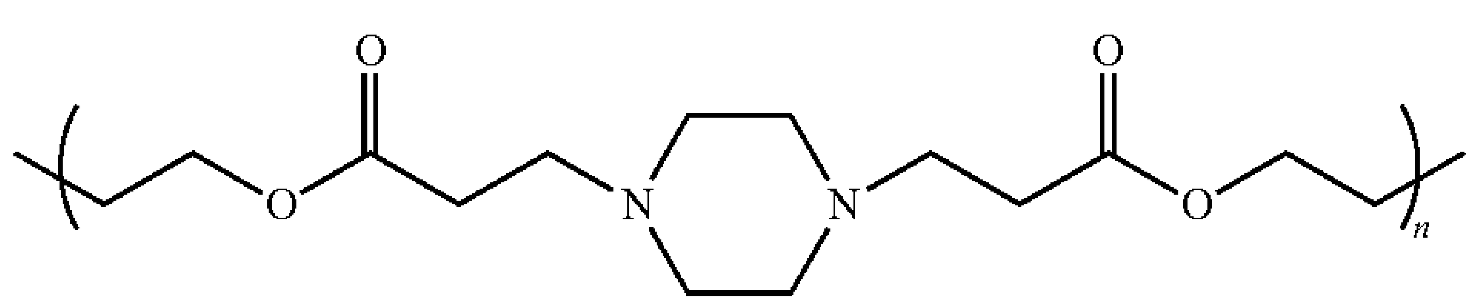
Representative Molecular Weight Data for Polymers 1-3.

| Polymer | Solvent | $M_n$[c] | PDI | Yield, % |
|---|---|---|---|---|
| 1[a] | THF | — | — | —[d] |
| 1[a] | $CH_2Cl_2$ | — | — | 82% |
| 2[a] | THF | 10.000 | 1.77 | 64% |
| 2[a] | $CH_2Cl_2$ | 17.500 | 2.15 | 75% |
| 3[a] | THF | 24.400 | 1.55 | 58% |
| 3[a] | $CH_2Cl_2$ | 30.800 | 2.02 | 70% |
| 1[b] | THF | 5.800 | 2.83 | 55% |

Poly-1



Poly-2



Poly-3

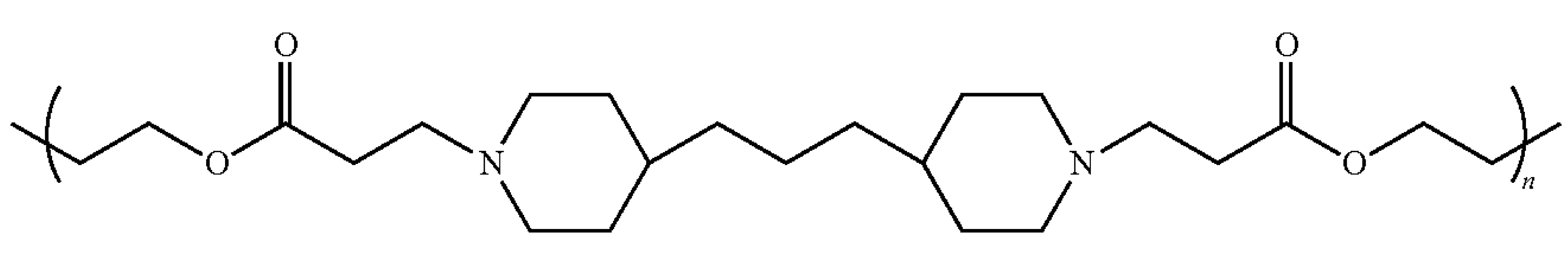


TABLE 1-continued

Representative Molecular Weight Data for Polymers 1-3.

| Polymer | Solvent | $M_n^c$ | PDI | Yield, % |
|---|---|---|---|---|
| $2^b$ | $CH_2Cl_2$ | 16.500 | 2.37 | 80%[e] |
| $3^b$ | $CH_2Cl_2$ | 31.200 | 2.55 | 86%[e] |

[a]Conditions: [diamine] = [1,4-butanediol diacrylate] = 0.38M, 50° C.,48 h.

[b]Conditions: [diamine] = [1,4-butanediol diacrylate] = 1.08M, 50° C, 48 h.

[c]GPC analysis was performed in THF/0.1M piperidine and molecular weights are reported versus polystyrene standards.

[d]No polymer was isolated under these conditions.

[e]The reaction solution became very viscous and eventually solidified under these conditions.

The structures of polymers 1-3 were confirmed by $^1H$ and $^{13}C$ NMR spectroscopy. These data indicate that the polymers were formed through the conjugate addition of the secondary amines to the acrylate moieties of 1,4-butanediol diacrylate and not through the formation of amide linkages under our reaction conditions. Additionally, the newly formed tertiary amines in the polymer backbones do not participate in subsequent addition reactions with diacrylate monomer, which would lead to branching or polymer crosslinking. This fortunate result appears to be unique to polymers of this type produced from bis(secondary amine) monomers. The synthesis of analogous polymers employing difunctional primary amines as monomers (such as 1,4-diaminobutane) may lead to polymer branching and the formation of insoluble crosslinked polymer networks if conditions are not explicitly controlled.

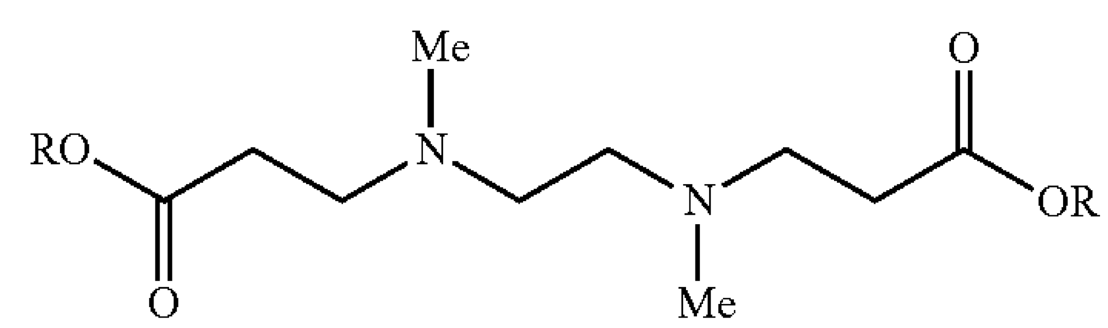
In view of the juxtaposition of amines and esters within the backbones of polymers 1-3, we were initially concerned that hydrolysis might occur too rapidly for the polymers to be of practical use. For example, poly(4-hydroxy-L-proline ester) and poly[α-(4-aminobutyl)-L-glycolic acid] degrade quite rapidly near neutral pH, having half lives of roughly 2 hr (Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; incorporated herein by reference) and 30 min (Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; incorporated herein by reference), respectively (Such rapid degradation times did not preclude the application of these polymers to gene delivery (See references, Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; each of which is incorporated herein by reference). However, extremely rapid degradation rates generally introduce additional concerns surrounding the manipulation, storage, and application of degradable polymers.). Analysis of polymers 1 and 2 by aqueous GPC using 1% acetic acid/water as eluent, however, revealed that degradation was sufficiently slow in acidic media. For example, the GPC traces of polymers 1 and 2 sampled under these conditions over a period of 4-5 hours revealed no changes in molecular weights or polydispersities (Polymer 3 could not be analyzed by aqueous GPC.). We were also concerned that significant backbone hydrolysis might occur during the isolation of the hydrochloride salts of polymers 1-3. To prevent hydrolysis during the protonation and isolation of these polymers, anhydrous solvents were employed and reactions were performed under an argon atmosphere. Analysis of the polymers before and after protonation revealed no observable hydrolysis. For example, the GPC trace of a sample of polymer 3 after precipitation from $CH_2Cl_2$ with 1.0 M diethyl ether/HCl ($M_n$=15,300; PDI=1.90) was virtually identical to the molecular weight of the polymer prior to protonation ($M_n$=15,700; PDI=1.92) and no lower molecular weight species were evident (Comparative GPC data were collected employing THF/0.1M piperidine as eluent (see Experimental Section above). The HCl salts of the polymers were insoluble in THF, but were soluble



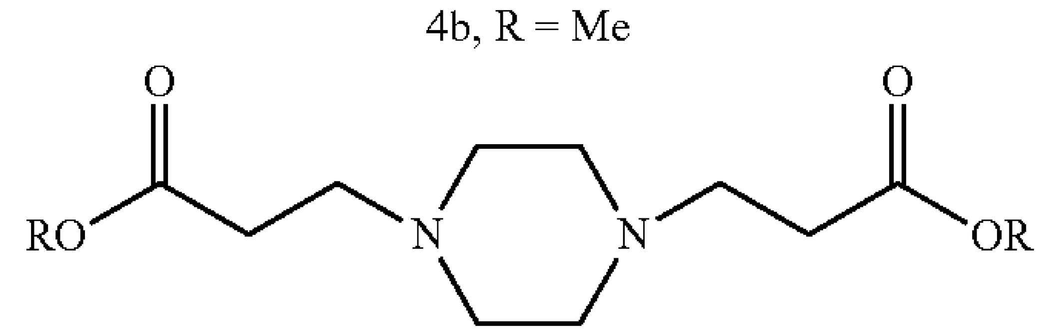
in THF/0.1 M piperidine concomitant with the production of a fine white precipitate which was filtered prior to injection.). Solid samples of polymers 1-3 could be stored for several months without detectable decreases in molecular weight.

Polymers 1-3 were specifically designed to degrade via hydrolysis of the ester bonds in the polymer backbones. However, an additional concern surrounding the overall stability and biocompatibility of these polymers is the potential for unwanted degradation to occur through retro-Michael reaction under physiological conditions. Because these polymers were synthesized via the Michael-type reaction of a secondary amine to an acrylate ester, it is possible that the polymers could undergo retro-Michael reaction to regenerate free acrylate groups, particularly under acidic conditions. Acrylate esters are potential DNA-alkylating agents and are therefore suspected carcinogens (for recent examples, see: Schweikl et al. Mutat. Res. 438:P71-P78, 1999; Yang et al. Carcinogenesis 19:P1117-P1125, 1998; each of which is incorporated herein by reference). Because these polymers are expected to encounter the reduced pH environment within the endosomal vesicles of cells (pH=5.0-5.5) during transfection, we addressed the potential for the degradation of these polymers to occur through a retro-Michael pathway.

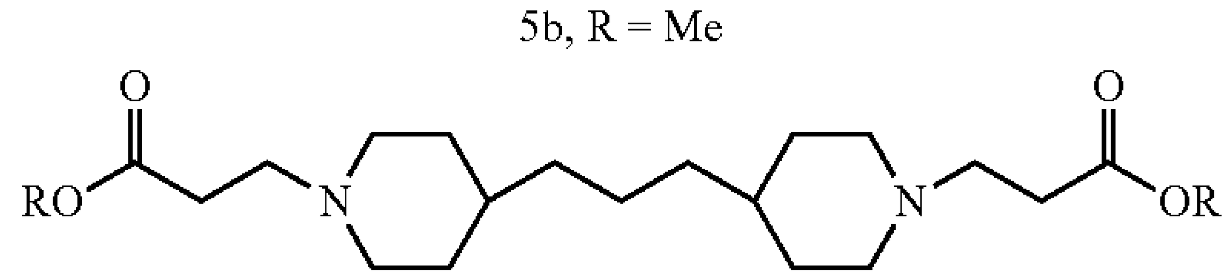
Under extremely acidic (pH<3) or basic (pH>12) conditions, polymers 1-3 degraded rapidly and exclusively to 1,4-butanediol and the anticipated bis(β-amino acid) byproducts 4a-6a as determined by $^1H$ NMR spectroscopy. No spectroscopic evidence for retro-Michael addition under these conditions was found. It is worth noting that bis(P-amino acid) degradation products 4a-6a would be less likely to undergo a retro-Michael reaction, as acrylic acids are generally less activated Michael addition partners (Perlmutter, P., in Conjugate Addition Reactions in Organic Synthesis, Pergamon Press, New York, 1992; incorporated herein by reference). Further degradation of compounds 4a-6a under these conditions was not observed.



4a, R = H
4b, R = Me



5a, R = H
5b, R = Me



5a, R = H
6b, R = Me

The kinetics of polymer degradation were investigated under the range of conditions likely to be encountered by these polymers during transfection. Degradation was monitored at 37° C. at buffered pH values of 5.1 and 7.4 in order to approximate the pH of the environments within endosomal vesicles and the cytoplasm, respectively. The hydrochloride salts of polymers 1-3 were added to the appropriate buffer, incubated at 37° C., and aliquots were removed at appropriate times. Aliquots were frozen immediately, lyophilized, and polymer was extracted into THF/0.1 M piperidine for analysis by GPC. FIG. **1** shows the degradation profiles of polymers 1-3 as a function of time. The polymers degraded more slowly at pH 5.1 than at pH 7.4. Polymers 1-3 displayed similar degradation profiles at pH 5.1, each polymer having a half-life of approximately 7-8 hours. In contrast, polymers 1 and 3 were completely degraded in less than 5 hours at pH 7.4. These results are consistent with the pH-degradation profiles of other amine-containing polyesters, such as poly(4-hydroxy-L-proline ester), in which pendant amine functionalities are hypothesized to act as intramolecular nucleophilic catalysts and contribute to more rapid degradation at higher pH (Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; each of which is incorporated herein by reference). While the possibility of intramolecular assistance cannot be ruled out, it is less likely for polymers 1-3 because the tertiary amines in these polymers should be less nucleophilic. The degradation of polymer 2 occurred more slowly at pH 7.4 than at pH 5.1 (FIG. **1**). This anomalous behavior is most likely due to the incomplete solubility of polymer 2 at pH 7.4 and the resulting heterogeneous nature of the degradation milieu (Polymers 2 and 3 are not completely soluble in water at pH 7.4. While polymer 3 dissolved relatively rapidly during the degradation experiment, solid particles of polymer 2 were visible for several days.

Cytotoxicity Assays

Poly(lysine) and PEI have been widely studied as DNA condensing agents and transfection vectors (Luo et al. Nat. Biotechnol. 18:33-37, 2000; Behr Acc. Chem. Res. 26:274-278, 1993; Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; Behr Chimia 51:34-36, 1997; Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Felgner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference) and are the standards to which new polymeric vectors are often compared (Putnam et al. Macromolecules 32:3658-3662, 1999;Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; Gonzalez et al. Bioconjugate Chem. 10:1068-1074, 1999; each of which is incorporated herein by reference). Unfortunately, as outlined above, these polymers are also associated with significant levels of cytotoxicity and high levels of gene expression are usually realized only at a substantial cost to cell viability. To determine the toxicity profile of polymers 1-3, a MTT/thiazolyl blue dye reduction assay using the NIH 3T3 cell line and the hydrochloride salts of polymers 1-3 was conducted as an initial indicators. The 3T3 cell line is commonly employed as a first level screening population for new transfection vectors, and the MTT assay is generally used as an initial indicator of cytotoxicity, as it determines the influences of added substances on cell growth and metabolism (Hansen et al. Immunol. Methods 119:203-210, 1989; incorporated herein by reference).

Cells were incubated with polymer 1 ($M_n$=5 800), polymer 2 ($M_n$=11 300), and polymer 3 ($M_n$=22 500) as described in the Experimental Section. As shown in FIG. **2**, cells incubated with these polymers remained 100% viable relative to controls at concentrations of polymer up to 100 μg/mL. These results compare impressively to data obtained for cell populations treated with PEI ($M_n$=25 000), included as a positive control for our assay as well as to facilitate comparison to this well-known transfection agent. Fewer than 30% of cells treated with PEI remained viable at a polymer concentration of 25 μg/mL, and cell viability was as low as 10% at higher concentrations of PEI under otherwise identical conditions. An analogous MTT assay was performed using independently synthesized bis(β-amino acid)s 4a-6a to screen the cytotoxicity of the hydrolytic degradation products of these polymers. (Bis(β-amino acid)s 4a-6a should either be biologically inert or possess mild or acute toxicities which are lower than traditional polycationic transfection vectors. In either case, the degradation of these materials should facilitate rapid metabolic clearance.). Compounds 4a-6a and 1,4butanediol did not perturb cell growth or metabolism in this initial screening assay (data not shown). A more direct structure/function-based comparison between polymers 1-3 and PEI cannot be made due to differences in polymer structure and molecular weight, both of which contribute to polycation toxicity. Nonetheless, the excellent cytotoxicity profiles of polymers 13 alone suggested that they were interesting candidates for further study as DNA condensing agents.

Self Assembly of Polymers 1-3 with Plasmid DNA

The tendency of cationic polyamines to interact electrostatically with the polyanionic backbone of DNA in aqueous solution is well known. Provided that the polymers are sufficiently protonated at physiological pH, and that the amines are sterically accessible, such interactions can result in a self-assembly process in which the positively and negatively charged polymers form well-defined conjugates (Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference). The majority of polyamines investigated as DNA-complexing agents and transfection vectors have incorporated amines at the terminal ends of short, conformationally flexible side chains (e.g., poly(lysine) and methacrylate/methacrylamide polymers) (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; van de Wetering et al. Bioconjugate Chem. 10:589-597, 1999; each of which is incorporated herein by reference), or accessible amines on the surfaces of spherical or globular polyamines (e.g., PEI and PAMAM dendrimers) (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; Kukowska-Latallo et al. Proc. Natl. Acad. Sci. USA 93:4897-4902, 1996; Tang et al. Bioconjugate Chem. 7:703-714, 1996; Haensler et al. Bioconjugate Chem. 4:372-379, 1993; each of which is incorporated herein by reference). Because polymers 1-3 contain tertiary amines, and those tertiary amines are located in a sterically crowded environment (flanked on two sides by the polymer backbones), we were initially concerned that the protonated amines might not be sufficiently able to interact intimately with DNA.

The ability of polymers 1-3 to complex plasmid DNA was demonstrated through an agarose gel shift assay. Agarose gel electrophoresis separates macromolecules on the basis of both charge and size. Therefore, the immobilization of DNA on an agarose gel in the presence of increasing concentrations of a polycation has been widely used as an assay to determine the point at which complete DNA charge neutralization is achieved (Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; Gonzalez et al. Bioconjugate Chem. 10: 1068-1074, 1999; each of which is incorporated herein by reference). As mentioned above, the hydrochloride salts of polymers 1-3 are soluble in water. However, polymers 2 and 3 are not completely soluble at pH 7.2 over the full range of desired polymer concentrations. Therefore, DNA/polymer complexes were prepared in MES buffer (25 mM, pH=6.0). DNA/polymer complexes were prepared by adding an aqueous solution of DNA to vortexing solutions of polymer in MES at desired DNA/polymer concentrations (see Experimental Section). The resulting DNA/polymer complexes remained soluble upon dilution in the electrophoresis running buffer (20 mM HEPES, pH=7.2) and data were obtained at physiological pH. As a representative example, FIG. **3** depicts the migration of plasmid DNA (pCMV-Luc) on an agarose gel in the presence of increasing concentrations of polymer 1.

As shown in FIG. **3**, retardation of DNA migration begins at DNA/1 ratios as low as 1:0.5 (w/w) and migration is completely retarded at DNA/polymer ratios above 1:1.0 (w/w) (DNA/polymer weight ratios rather than DNA/polymer charge ratios are reported here. Although both conventions are used in the literature, we find weight ratios to be more practical and universal, since the overall charge on a polyamine is subject to environmental variations in pH and temperature. While DNA/polymer charge ratios are descriptive for polymers such as poly(lysine), they are less meaningful for polymers such as PEI and 1-3 which incorporate less basic amines.). Polymers 2 and 3 completely inhibit the migration of plasmid DNA at DNA/polymer ratios (w/w) above 1:10 and 1:1.5, respectively (data not shown). These results vary markedly from gel retardation experiments conducted using model "monomers." Since the true monomers and the degradation products of polymers 1-3 do not adequately represent the repeat units of the polymers, we used bis(methyl ester)s 4b-6b to examine the extent to which the polyvalency and cooperative binding of polycations 1-3 is necessary to achieve DNA immobilization. "Monomers" 4b-6b did not inhibit the migration of DNA at DNA/"monomer" ratios (w/w) of up to 1:30 (data not shown).

The reasons for the less-efficient complexation employing polymer 2 in the above gel electrophoresis assays most likely results from differences in the $pK_a$ values of the amines in these polymers. The direct measurement of the $pK_a$ values of polymers 1-3 is complicated by their degradability. However, we predict the range of $pK_a$ values of the amines in polymers 1 and 2 to extend from approximately 4.5 and 8.0 for polymer 1, to 3.0 and 7.0 for polymer 2, based on comparisons to structurally related poly(β-amino amides) (The $pK_a$ values of structurally-related poly(β-amino amides) containing piperazine and dimethylethylene diamine units in their backbones have been reported. Barbucci et al. Polymer 21:81-85, 1980; Barbucci et al. Polymer 19:1329-1334, 1978; Barbucci et al. Macromolecules 14:1203-1209, 1981; each of which is incorporated herein by reference). As a result, polymer 2 should be protonated to a lesser extent than polymer 1 at physiological or near-neutral pH, and would therefore be a less effective DNA condensing agent. The range of $pK_a$ values for polymer 3 should be higher than the range for polymers 1 and 2 due to the increased distance between the nitrogen atoms. Accordingly, polymer 3 forms complexes with DNA at substantially reduced concentrations relative to polymer 2.

Agarose gel retardation assays are useful in determining the extent to which polycations interact with DNA. To be useful transfection agents, however, polycations must also be able to self-assemble plasmid DNA into polymer/DNA complexes small enough to enter a cell through endocytosis. For most cell types, this size requirement is on the order of 200 nm or less (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; incorporated herein by reference), although larger particles can also be accomodated (Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Felgner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference). The ability of polymers 1-3 to compact plasmid DNA into nanometer-sized structures was determined by quasi-elastic laser light scattering (QELS), and the relative surface charges of the resulting complexes were quantified through ζ-potential measurements. DNA/polymer particles used for particle sizing and 1-potential measurements were formed as described above for agarose gel electrophoresis assays and diluted in 20 mM HEPES buffer (pH=7.0) for analysis, as described in the Experimental Section.

Polymer 1 formed complexes with diameters ranging from 90-150 nm at DNA/polymer ratios above 1:2 (w/w), and polymer 2 condensed DNA into particles on the order of 60-125 nm at DNA/polymer ratios above 1:10. These results are consistent with the data obtained from agarose gel electrophoresis experiments above. However, the particles in these experiments aggregated over a period of hours to yield larger complexes with diameters in the range of 1-2 microns. The tendency of these particles to aggregate can be rationalized by the low ζ-potentials of the DNA/polymer particles observed under these conditions. For example, complexes formed from polymer 1 at DNA/polymer ratios above 1:10 had average ζ-potentials of +4.51 (±10.50) mV. The ζ-potentials of complexes formed from polymer 2 at DNA/polymer ratios above 1:20 were lower, reaching a limiting value of +1.04 (±10.57) mV. These differences correlate with the estimated $pK_a$ values for these polymers, as the surfaces of particles formed from polymer 1 would be expected to slightly more protonated than particles formed from polymer 2 at pH=7.0.

Polymer 3 formed complexes with diameters in the range of 50-150 nm at DNA/polymer ratios above 1:2. As a representative example, FIG. **4** shows the average effective diameters of particles formed with polymer 3 as a function of polymer concentration. The diameters of the particles varied within the above range from experiment to experiment under otherwise identical conditions, possibly due to subtle differences during the stirring or addition of DNA solutions during complex formation (The order of addition of polymer and DNA solutions had considerable impact on the nature of the resulting DNA/polymer complexes. In order to form small particles, for example, it was necessary to add the DNA solution to a vortexing solution of polymer. For cases in which polymer solutions were added to DNA, only large micron-sized aggregates were observed. Thus, it is possible that subtle differences in stirring or rate of addition could be responsible for variation in particle size). The ζ-potentials for complexes formed from polymer 3 were on the order of +10 to +15 mV at DNA/polymer ratios above 1:1, and the complexes did not aggregate extensively over an 18 hour period (pH=7, 25° C.) The positive ζ-potentials of these complexes may be significant beyond the context of particle stability, as net positive charges on particle surfaces may play a role in triggering endocytosis (Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; Behr Chimia 51:34-36, 1997; Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Feigner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference).

Particles formed from polymer 3 were also relatively stable at 37° C. For example, a sample of DNA/3 (DNA/3=1:5, average diameter=83 nm) was incubated at 37° C. for 4 hours. After 4 hours, a bimodal distribution was observed consisting of a fraction averaging 78 nm (>98% by number, 70% by volume) and a fraction of larger aggregates with average diameters of approximately 2.6 microns. These results suggest that the degradation of complexes formed from polymer 3 occurred more slowly than the degradation of polymer in solution, or that partial degradation did not significantly affect the stability of the particles. The apparently increased stability of DNA/polymer complexes formed from degradable polycations relative to the degradation of the polymers in solution has also been observed for DNA/polymer complexes formed from poly(4-hydroxy-L-proline ester) (Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; incorporated herein by reference).

The particle size and ζ-potential data in FIGS. **4** and **5** are consistent with models of DNA condensation observed with other polycations (Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; Putnam et al. Macromolecules 32:3658-3662, 1999; Lim et al. J. Am. Chem. Soc. 121:5633-5639, 1999; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; Gonzalez et al. Bioconjugate Chem. 10:1068-1074, 1999; each of which is incorporated herein by reference). DNA is compacted into small negatively charged particles at very low polymer concentrations and particle sizes increase with increasing polymer concentration (Accurate light scattering data could not be obtained for DNA alone or for DNA/polymer associated species at DNA/polymer ratios lower than 1:0.5, since flexible, uncondensed DNA does not scatter light as extensively as compacted DNA (Kabanov et al., in Self-Assembling Complexes for Gene Delivery. From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; incorporated herein by reference).). Complexes reach a maximum diameter as charge neutrality is achieved and aggregation occurs. Particle sizes decrease sharply at DNA/polymer concentrations above charge neutrality up to ratios at which additional polymer does not contribute to a reduction in particle diameter. This model is confirmed by ζ-potential measurements made on complexes formed from these polymers. As shown in FIG. **5**, the ζ-potentials of polymer/DNA particles formed from polymer 3 were negative at low polymer concentrations and charge neutrality was achieved near DNA/polymer ratios of 1:0.75, resulting in extensive aggregation. The ζ-potentials of the particles approached a limiting value ranging from +10 to +15 mV at DNA/polymer ratios above 1:2.

The average diameters of the complexes described above fall within the general size requirements for cellular endocytosis. We have initiated transfection experiments employing the NIH 3T3 cell line and the luciferase reporter gene (pCMV-Luc). Thus far, polymers 1 and 2 have shown no transfection activity in initial screening assays. By contrast, polymer 3 has demonstrated transfection efficiencies exceeding those of PEI under certain conditions. Transfection experiments were performed according to the following general protocol: Cells were grown in 6-well plates at an initial seeding density of 100,000 cells/well in 2 mL of growth medium. Cells were grown for 24 hours after which the growth medium was removed and replaced with 2 mL of serum-free medium. DNA/polymer complexes were formed as described in the Experimental Section (2 μg DNA, DNA/3=1:2 (w/w), 100 μL in MES (pH=6.0)] and added to each well. DNA/PEI complexes were formed at a weight ratio of 1:0.75, a ratio generally found in our laboratory to be optimal for PEI transfections. Transfections were carried out in serum-free medium for 4 hours, after which medium was replaced with growth medium for 20 additional hours. Relative transfection efficiencies were determined using luciferase (Promega) and cell protein assay (Pierce) kits. Results are expressed as relative light units (RLU) per mg of total cell protein: for complexes of polymer 3, 1.07 (±0.43)$\times 10^6$ RLU/mg; for PEI complexes, 8.07 (±0.16)$\times 10^5$ RLU/mg). No luciferase expression was detected for control experiments employing naked DNA or performed in the absence of DNA. These transfection data are the results of initial screening experiments. These data suggest that polymers of this general structure hold promise as synthetic vectors for gene delivery and are interesting candidates for further study. The relative efficacy of polymer 3 relative to PEI is interesting, as our initial screening experiments were performed in the absence of chloroquine and polymer 3 does not currently incorporate an obvious means of facilitating endosomal escape. It should be noted, however, that the $pK_a$ values of the amines in these polymers can be "tuned" to fall more directly within the range of physiologically relevant pH using this modular synthetic approach. Therefore, it will be possible to further engineer the "proton sponge" character (Behr Chimia 51:34-36, 1997; Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Felgner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference) of these polymers, and thus enhance their transfection efficacies, directly through the incorporation of or copolymerization with different diamine monomers.

## SUMMARY

A general strategy for the preparation of new degradable polymeric DNA transfection vectors is reported. Poly(β-amino esters) 1-3 were synthesized via the conjugate addition of N,N'-dimethylethylenediamine, piperazine, and 4,4'-trimethylenedipiperidine to 1,4-butanediol diacrylate. The amines in the bis(secondary amine) monomers actively participate in bond-forming processes during polymerization, obviating the need for amine protection/deprotection processes which characterize the synthesis of other poly(amino esters). Accordingly, this approach enables the generation of a variety of structurally diverse polyesters containing tertiary amines in their backbones in a single step from commercially available staring materials. Polymers 1-3 degraded hydrolytically in acidic and alkaline media to yield 1,4-butanediol and β-amino acids 4a-6a and the degradation kinetics were investigated at pH 5.1 and 7.4. The polymers degraded more rapidly at pH 7.4 than at pH 5.1, consistent with the pH/degradation profiles reported for other poly(amino esters). An initial screening assay designed to determine the effects of polymers 1-3 on cell growth and metabolism suggested that these polymers and their hydrolytic degradation products were non-cytotoxic relative to PEI, a non-degradable cationic polymer conventionally employed as a transfection vector.

Polymers 1-3 interacted electrostatically with plasmid DNA at physiological pH, initiating self-assembly processes that resulted in nanometer-scale DNA/polymer complexes. Agarose gel electrophoresis, quasi-elastic dynamic light scattering (QELS), and zeta potential measurements were used to determine the extent of the interactions between the oppositely charged polyelectrolytes. All three polymers were found to condense DNA into soluble DNA/polymer particles

on the order of 50-200 nm. Particles formed from polymers 1 and 2 aggregated extensively, while particles formed from polymer 3 exhibited positive ζ-potentials (e.g., +10 to +15 mV) and did not aggregate for up to 18 hours. The nanometer-sized dimensions and reduced cytotoxicities of these DNA/polymer complexes suggest that polymers 1-3 may be useful as degradable polymeric gene transfection vectors. A thorough understanding of structure/activity relationships existing for this class of polymer will expedite the design of safer polymer-based alternatives to viral transfection vectors for gene therapy.

Example 2

Rapid, pH-Triggered Release from Biodegradable Poly(β-Amino Ester) Microspheres Within the Ranger of Intracellular pH

Experimental Section

Fabrication of microspheres. The optimized procedure for the fabrication of microspheres was conducted in the following general manner: An aqueous solution of rhodamine-conjugated dextran (200 μL of a 10 μg/μL solution, $M_n$≈70 kD) was suspended in a solution of poly-1 in $CH_2Cl_2$ (200 mg of poly-1 in 4 mL $CH_2Cl_2$, $M_n$≈10 kD), and the mixture was sonicated for 10 seconds to form a primary emulsion. The cloudy pink emulsion was added directly to a rapidly homogenized (5,000 rpm) solution of poly(vinyl alcohol) [50 mL, 1% PVA (w/w)] to form the secondary emulsion. The secondary emulsion was homogenized for 30 seconds before adding it to a second aqueous PVA solution [100 mL, 0.5% PVA (w/w)]. Direct analysis of the microsphere suspension using a Coulter microparticle analyzer revealed a mean particle size of approximately 5 micrometers. The secondary emulsion was stirred for 2.5 hours at room temperature, transferred to a cold room (4° C.), and stirred for an additional 30 minutes. Microspheres were isolated at 4° C. via centrifugation, resuspended in cold water, and centrifuged again to remove excess PVA. The spheres were resuspended in water (15 mL) and lyophilized to yield a pink, fluffy powder. Characterization of the lyophilized microspheres was performed by optical, fluorescence, and scanning electron microscopies as described. Zeta potential was determined using a Brookhaven Instruments ZetaPALS analyzer.

Discussion

Microparticles formed from biodegradable polymers are attractive for use as delivery devices, and a variety of polymer-based microspheres have been employed for the sustained release of therapeutic compounds (Anderson Nature 392(Suppl.):25-30, 1996; Friedman Nature Med. 2:144-147, 1996; Crystal Science 270:404-410, 1995; Mulligan Science 260:926-932, 1993; Luo et al. Nat. Biotechnol. 18:33-37, 2000; Behr Acc. Chem. Res. 26:274-278, 1993; each of which is incorporated herein by reference). However, for small-molecule-, protein-, and DNA-based therapeutics that require intracellular administration and trafficking to the cytoplasm, there is an increasing demand for new materials that facilitate triggered release in response to environmental stimuli such as pH (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; incorporated herein by reference). Following endocytosis, the pH within cellular endosomal compartments is lowered, and foreign material is degraded upon fusion with lysosomal vesicles (Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; incorporated herein by reference). New materials that release molecular payloads upon changes in pH within the intracellular range and facilitate escape from hostile intracellular environments could have a fundamental and far-reaching impact on the administration of hydrolytically- and/or enzymatically-labile drugs (Zauner et al. Adv. Drug Del. Rev. 30:97-113, 1998; Kabanov et al. Bioconjugate Chem. 6:7-20, 1995; each of which is incorporated herein by reference). Herein, the fabrication of pH-responsive polymer micro spheres that release encapsulated contents quantitatively and essentially instantaneously upon changes in pH within the intracellular range is reported.

The synthesis of poly(β-amino ester) 1 has been described above in Example 1 (Miller Angew. Chem. Int. Ed. 37:1768-1785, 1998; Hope et al. Molecular Membrane Technology 15: 120 14, 1998; Deshmukh et al. New J. Chem. 21:113-124, 1997; each of which is incorporated herein by reference). Poly-1 is hydrolytically degradable, was non-cytotoxic in initial screening assays, and is currently under investigation as a synthetic vector for DNA delivery in gene therapy applications. The solubility of the polymer in aqueous media is directly influenced by solution pH. Specifically, the solid, unprotonated polymer is insoluble in aqueous media in the pH range 7.0 to 7.4, and the transition between solubility and insolubility occurs at a pH around 6.5. Based on the differences between extracellular and endosomal pH (7.4 and 5.0-6.5, respectively), we hypothesized that microspheres formed from poly-1 might be useful for the encapsulation and triggered release of compounds within the range of intracellular pH.

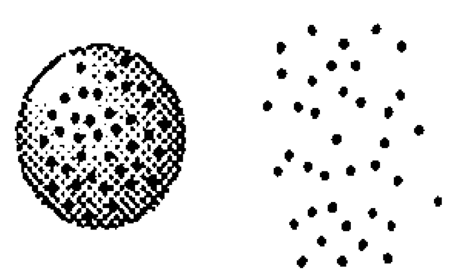
The encapsulation of therapeutic compounds within polymer microspheres is often achieved employing a double emulsion process (O'Donnell et al. Adv. Drug Delivery Rev. 28:25-42, 1997; incorporated herein by reference). The double emulsion process is well established for the fabrication of microspheres from hydrophobic polymers such as poly(lactic-co-glycolic acid) (PLGA), a biodegradable polymer conventionally employed in the development of drug delivery devices (Anderson et al. Adv. Drug Delivery Rev. 28:5-24, 1997; Okada Adv. Drug Delivery Rev. 28:43-70, 1997; each of which is incorporated herein by reference). Preliminary experiments demonstrated the feasibility of the double emulsion process for the encapsulation of water-soluble compounds using poly-1. Rhodamine-conjugated dextran was chosen as a model for subsequent encapsulation and release studies for several reasons: 1) rhodamine is fluorescent, allowing loading and release profiles to be determined by fluorescence spectroscopy, 2) loaded microspheres could be imaged directly by fluorescence microscopy, and 3) the fluorescence intensity of rhodamine is relatively unaffected by pH within the physiological range (Haugland, Handbook of Fluorescent Probes and Research Chemicals, 6th ed., Molecular Probes, Inc., 1996, p. 29; incorporated herein by reference).

Microspheres encapsulating labeled dextran were fabricated from poly-1 and compared to controls formed from PLGA. The size distributions of microspheres formed from poly-1 correlated well with the distributions of PLGA microspheres within the range of 5-30 μm. Average particle sizes could be controlled by variations in experimental parameters such as homogenization rates and aqueous/organic solvent ratios (O'Donnell et al. Adv. Drug Delivery Rev. 28:25-42, 1997; incorporated herein by reference). In contrast to PLGA microspheres, however, spheres formed from poly-1 aggregated extensively during centrifugation and washing steps (see Experimental Section above). Microspheres resuspended at pH 7.4 consisted primarily of large aggregates, and scanning electron microscopy (SEM) images revealed clusters of spheres that appeared to be physically joined or "welded" (data not shown).

It was found that aggregation could be eliminated if centrifugation and washing were conducted at reduced temperatures (4° C.), presumably due to the hardening of the polymer spheres at this lower temperature. SEM images of dextran-loaded poly-1 microspheres prepared in the 8-10 μm range revealed significant fracturing and the formation of large holes on their surfaces. Microspheres targeted in the range of 4-6 μm, however, were essentially free of cracks, holes, and other defects (FIG. **6**). Microspheres formulated for subsequent release experiments were fabricated in the smaller (<6 μm) range. Encapsulation efficiencies for loaded poly-1 microspheres, determined by dissolving the spheres at pH 5.1 and measuring fluorescence intensity, were as high as 53%.

Suspensions of dried poly-1 microspheres at pH=7.4 consisted primarily of single, isolated microspheres as determined by optical and fluorescence microscopy (FIG. **8**a). The zeta potential (ζ) of microparticle suspensions of poly-1 microspheres at pH 7 was +3.75 (+0.62) mV, suggesting that the surfaces of the microspheres carry an overall positive charge at physiological pH. This could be relevant to the targeting of these microspheres for cellular uptake, because net positive charges on particle surfaces may play a role in triggering endocytosis (Zauner et al. Adv. Drug Delivery Rev. 30:97-113, 1998; incorporated herein by reference).

Poly-1 microspheres suspended at pH 7.4 remained stable toward aggregation and degradation for several weeks (by visual inspection), but the microspheres dissolved instantly when the pH of the suspending medium was lowered between 5.1 and 6.5.



pH 7.4 → pH <6.5

The release of labeled dextran from poly-1 microspheres was determined quantitatively by fluorescence microscopy (FIG. **7**). The release profile at pH 7.4 was characterized by a small initial burst in fluorescence (7-8%) which reached a limiting value of about 15% after 48 hours. This experiment demonstrated that the degradation of poly-1 was relatively slow under these conditions and that greater than 90% of encapsulated material could be retained in the polymer matrix for suitably long periods of time at physiological pH.

To examine the application of poly-1 microspheres to the triggered release of encapsulated drugs in the endosomal pH range, we conducted a similar experiment in which the pH of the suspension medium was changed from 7.4 to 5.1 during the course of the experiment. As shown in FIG. **7**, the microspheres dissolved rapidly when the suspension buffer was exchanged with acetate buffer (0.1 M, pH=5.1), resulting in essentially instantaneous and quantitative release of the labeled dextran remaining in the polymer matrices. In sharp contrast, the release from dextran-loaded PLGA microspheres did not increase for up to 24 hours after the pH of the suspending medium was lowered (FIG. **7**). FIG. **8** shows fluorescence microscopy images of: (a) a sample of dextran-loaded microspheres at pH 7.4; and (b) a sample to which a drop of acetate buffer was added at the upper right edge of the microscope coverslip. The rapid release of rhodamine-conjugated dextran was visualized as streaking extending from the dissolving microspheres in the direction of the diffusion of added acid and an overall increase in background fluorescence (elapsed time≈5 seconds).

When targeting therapeutic compounds for intracellular delivery via endocytosis or phagocytosis, it is not only important to consider a means by which the drug can be released from its carrier, but also a means by which the drug can escape endosomal compartments prior to being routed to lysosomal vesicles (Luo et al. Nat. Biotechnol. 18:33-37, 2000; Zauner et al. Adv. Drug Delivery Rev. 30:97-113, 1998; each of which is incorporated herein by reference). One strategy for facilitating endosomal escape is the incorporation of weak bases, or "proton sponges," which are believed to buffer the acidic environment within an endosome and disrupt endosomal membranes by increasing the internal osmotic pressure within the vesicle (Demeneix et al., in Artificial Self-Assembling Systems for Gene Delivery (Felgner et al., Eds.), American Chemical Society, Washington, D.C., 1996, pp. 146-151; incorporated herein by reference). Poly-1 microspheres are capable of releasing encapsulated material in the endosomal pH range via a mechanism (dissolution) that involves the protonation of amines in the polymer matrix. Thus, in addition to the rapid release of drug, poly-1 microspheres may also provide a membrane-disrupting means of endosomal escape, enhancing efficacy by prolonging the lifetimes of hydrolytically unstable drugs contained in the polymer matrix.

Microspheres fabricated from poly-1 could represent an important addition to the arsenal of pH-responsive materials applied for intracellular drug delivery, such as pH-responsive polymer/liposome formulations (Gerasimov et al. Adv. Drug Delivery Rev. 38:317-338, 1999; Linhart et al. Langmuir 16:122-127, 2000; Linhardt et al. Macromolecules 32:4457-4459, 1999; each of which is incorporated herein by reference). In contrast to many liposomal formulations, polymer microspheres are physically robust and can be stored dried for extended periods without deformation, decomposition, or degradation (Okada Adv. Drug Delivery Rev. 28:43-70, 1997; incorporated herein by reference)—an important consideration for the formulation and packaging of new therapeutic delivery systems. The microspheres investigated in this current study fall within the size range of particles commonly used to target delivery to macrophages (Hanes et al. Adv. Drug Delivery Rev. 28:97-119, 1997; incorporated herein by reference). The rapid pH-release profiles for the poly-1 microspheres described above may therefore be useful in the design of new DNA-based vaccines which currently employ PLGA as an encapsulating material (Singh et al. Proc. Natl. Acad. Sci. USA 97:811-816, 2000; Andoet al. J. Pharm. Sci. 88:126-130, 1999; Hedley et al. Nat. Med. 4:365-368, 1998; each of which is incorporated herein by reference).

## Example 3

### Accelerated Discovery of Synthetic Transfection Vectors: Parallel Synthesis and Screening of a Degradable Polymer Library

Introduction

The safe and efficient delivery of therapeutic DNA to cells represents a fundamental obstacle to the clinical success of gene therapy (Luo et al. Nat. Biotechnol. 18:33-37, 2000; Anderson Nature 392 Suppl.:25-30, 1996; each of which is incorporated herein by reference). The challenges facing synthetic delivery vectors are particularly clear, as both cationic polymers and liposomes are less effective at mediating gene transfer than viral vectors. The incorporation of new design criteria has led to recent advances toward functional delivery systems (Lim et al. J. Am. Chem. Soc. 123:2460-2461, 2001; Lim et al. J. Am. Chem. Soc. 122:6524-6525, 2000; Hwang et al. Bioconjugate Chem. 12:280-290, 2001; Putnam et al. Proc. Natl. Acad. Sci. USA 98:1200-1205, 2001; Benns et al. Bioconjugate Chem. 11:637-645, 2000; Midoux et al. Bioconjugate Chem. 10:406-411, 1999; each of which is incorporated herein by reference). However, the paradigm for the development of polymeric gene delivery vectors remains the incorporation of these design elements into materials as part of an iterative, linear process—an effective, albeit slow, approach to the discovery of new vectors. Herein, we report a parallel approach suitable for the synthesis of large libraries of degradable cationic polymers and oligomers and the discovery of new synthetic vector families through rapid cell-based screening assays (for a report on the parallel synthesis and screening of degradable polymers for tissue engineering, see: Brocchini et al. J. Am. Chem. Soc. 119:4553-4554, 1997; incorporated herein by reference).

Experimental Section

General Considerations. All manipulations involving live cells or sterile materials were performed in a laminar flow hood using standard sterile technique. Gel permeation chromatography (GPC) was performed using a Hewlett Packard 1100 Series isocratic pump, a Rheodyne Model 7125 injector with a 100-μL injection loop, and two PL-Gel mixed-D columns in series (5 μm, 300×7.5 mm, Polymer Laboratories, Amherst, Mass.). THF/0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, Calif.) and processed using the TriSEC GPC software package (Viscotek Corporation, Houston, Tex.). The molecular weights and polydispersities of the polymers are reported relative to monodisperse polystyrene standards.

Materials. Primary amine and secondary amine monomers 1-20 were purchased from Aldrich Chemical Company (Milwaukee, Wis.), Lancaster (Lancashire, UK), Alfa Aesar Organics (Ward Hill, Mass.), and Fluka (Milwaukee, Wis.). Diacrylate monomers A-G were purchased from Polysciences, Inc. (Warrington, Pa.), Alfa Aesar, and Scientific Polymer Products, Inc. (Ontario, N.Y.). All monomers were purchased in the highest purity available (from 97% to 99+%) and were used as received without additional purification. Plasmid DNA containing the firefly luciferase reporter gene (pCMV-Luc) was purchased from Elim Biopharmaceuticals, Inc. (San Francisco, Calif.) and used without further purification. (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma Chemical Company (St. Louis, Mo.). Monkey kidney fibroblasts (COS-7 cells) used in transfection assays were purchased from American Type Culture Collection (Manassas, Va.) and grown at 37° C., 5% $CO_2$ in Dulbecco's modified Eagle's medium, 90%; fetal bovine serum, 10%; penicillin, 100 units/mL; streptomycin, 100 μg/mL. Luciferase detection kits used in high-throughput transfection assays were purchased from Tropix (Bedford, Mass.). All other materials and solvents were used as received without additional purification.

Synthesis of Polymer Library. All 140 polymerization reactions were conducted simultaneously as an array of individually labeled vials according to the following general protocol. Individual exceptions are noted where appropriate. Amine monomers 1-20 (2.52 mmol) were charged into appropriately labeled vials (as shown below): liquid monomers were measured and transferred quantitatively using microliter pipettes; solid monomers were weighed directly into each vial. Anhydrous $CH_2Cl_2$ (1 mL) was added to each vial. One equivalent of liquid diacrylates A-F (2.52 mmol) was added to each appropriate reaction vial using a microliter pipette, and the vial was capped tightly with a Teflon-lined cap. One equivalent of semi-solid diacrylate G was added to the appropriate vials as a solution in $CH_2Cl_2$ (2.52 mmol, 1 mL of a 2.52M solution in $CH_2Cl_2$) and the vials were tightly capped. An additional aliquot of $CH_2Cl_2$ (2 mL) was added to the reaction vials containing 19 and 20 to aid in the solubility of these monomers. The tightly capped vials were arrayed in two plastic test tube racks and secured to an orbital shaker in a 45° C. oven. (CAUTION: The heating of capped vials represents a possible explosion hazard. Oven temperature was monitored periodically for one week prior to the experiment to ensure reliable thermal stability. Temperatures were found to vary within +/−1° C. during this time period. Several test vials were monitored prior to conducting the larger experiment). The reaction vials were shaken vigorously at 45° C. for 5 days and allowed to cool to room temperature. Vials were placed in a large dessicator and placed under aspirator vacuum for 1 day and high vacuum for an additional 5 days to ensure complete removal of solvent. The samples obtained were analyzed by GPC (55% of total library, see Table 2) and used directly in all subsequent screening experiments.

TABLE 2

GPC survey of 55% of the screening library showing molecular weights ($M_W$) and polydispersities (shown in parentheses).

| | A | B | C | D | E | F | G |
|---|---|---|---|---|---|---|---|
| 1 | 5900 (1.93) | 4725 (1.89) | | | 5220 (1.95) | 1690 (1.74) | |
| 2 | 6920 (1.87) | 6050 (1.78) | | | 5640 (1.85) | | |
| 3 | 6690 (1.79) | 6050 (1.78) | | | | 2060 (1.76) | |
| 4 | 7810 (2.49) | 5720 (2.20) | 9720 (2.49) | 7960 (4.08) | 7940 (3.25) | | |
| 5 | 10 800 (2.75) | 5000 (2.50) | 15 300 (3.17) | 17 200 (6.91) | 15 300 (3.92) | Insol. | 9170 (2.50) |
| 6 | 21 000 (3.70) | 10 200 (3.4) | | 18 000 (6.06) | | | |
| 7 | 14 300 (3.25) | 11 880 (3.3) | 20 200 (3.44) | 10 300 (4.26) | 15 500 (4.89) | | 22 500 (3.92) |
| 8 | 2310 (1.62) | 11 520 (3.60) | | 2230 (1.73) | | | |
| 9 | 1010 (1.33) | 2505 (1.67) | 1240 (1.16) | | | Insol. | |
| 10 | | <1000 | Insol. | | | | |
| 11 | 6800 (1.91) | Insol. | 9440 (1.79) | 5550 (2.23) | 6830 (1.93) | 1990 (1.43) | 6420 (1.75) |
| 12 | 9310 (2.06) | 9100 (2.53) | 11 900 (2.18) | 5810 (1.77) | | | 12 300 (1.85) |
| 13 | 2990 (1.64) | 3180 (2.12) | 3680 (1.64) | 2550 (1.82) | 3230 (1.82) | | 3580 (1.64) |
| 14 | 1350 (1.35) | 3180 (2.12) | 2110 (1.69) | 1400 (1.4) | 1752 (1.46) | | 2025 (1.62) |
| 15 | | 1550 (1.51) | | | | | |
| 16 | | 16 380 (2.60) | | | | | |
| 17 | | 8520 (2.13) | | 7290 (1.94) | | | |
| 18 | | <1000 | | | | | |
| 19 | 12 400 (2.28) | 18 445 (2.17) | 39 700 (1.90) | 17 400 (1.93) | 14 800 (1.98) | | 13 900 (1.86) |
| 20 | 16 900 (2.40) | 46 060 (3.29) | 49 600 (2.25) | 30 700 (2.72) | 18 700 (2.72) | | 17 100 (2.22) |

Determination of Water Solubility. The solubilities of all samples sample were determined simultaneously at a concentration of 2 mg/mL in the following general manner. Each polymer sample (5 mg) was weighed into a 12 mL scintillation vial and 2.5 mL of acetate buffer (25 mM, pH=5.0) was added to each sample using an a pipettor. Samples were shaken vigorously at room temperature for 1 hour. Each sample was observed visually to determine solubility.

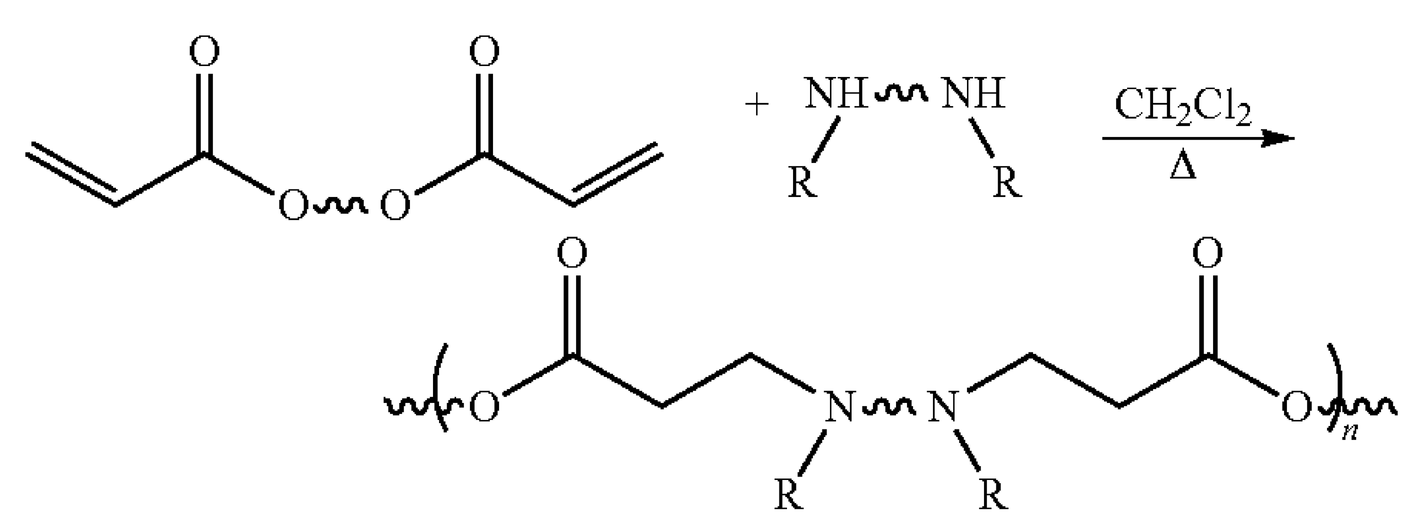
Agarose Gel Electrophoresis Assay. The agarose gel electrophoresis assay used to determine the ability of polymers to form complexes with DNA was performed in the following manner. Using the solutions prepared in the above solubility assay (2 mg/mL in acetate buffer, 25 mM, pH=5.0), stock solutions of the 70 water-soluble polymers were arrayed into a 96-well cell culture plate. DNA/polymer complexes were formed at a ratio of 1:5 (w/w) by transferring 10 μL of each polymer solution from the stock plate to a new plate using a multichannel pipettor. Each polymer was further diluted with 90 μL of acetate buffer (25 mM, pH=5.0, total volume=100 μL) and the plate was shaken for 30 seconds on a mechanical shaker. An aqueous solution of plasmid DNA (100 μL of a 0.04 μg/μL solution) was added to each well in the plate and the solutions were vigorously mixed using a multichannel pipettor and a mechanical shaker. DNA/polymer complexes were formed at a ratio of 1:20 (w/w) in the same manner with the following exceptions: 40 μL of polymer stock solution was transferred to a new plate and diluted with 60 μL of acetate buffer (total volume=100 μL) prior to adding the aqueous DNA solution (100 μL). DNA/polymer complexes were incubated at room temperature for 30 minutes, after which samples of each solution (15 μL) were loaded into a 1% agarose gel (HEPES, 20 mM, pH=7.2, 500 ng/mL ethidium bromide) using a multichannel pipettor. NOTE: Samples were loaded on the gel with a loading buffer consisting of 10% Ficoll 400 (Amersham Pharmacia Biotech, Uppsala, Sweden) in HEPES (25 mM, pH=7.2). Bromphenol blue was not included as a visual indicator in the loading buffer, since this charged dye appeared to interfere with the complexation of polymer and DNA. Samples were loaded according to the pattern shown in FIG. **9**, such that samples corresponding to DNA/polymer ratios of 1:5 and 1:20 were assayed in adjacent positions on the gel. The gel was run at 90V for 30 minutes and DNA bands were visualized by ethidium bromide staining.

General Protocol for Cell Transfection Assays. Transfection assays were performed in triplicate in the following general manner. COS-7 cells were grown in 96-well plates at an initial seeding density of 15,000 cells/well in 200 μL of phenol red-free growth medium (90% Dulbecco's modified Eagle's medium, 10% fetal bovine serum, penicillin 100 units/mL, streptomycin 100 μg/mL). Cells were grown for 24 hours in an incubator, after which the growth medium was removed and replaced with 200 μL of Optimem medium (Invitrogen Corp., Carlsbad, Calif.) supplemented with HEPES (total concentration=25 mM). Polymer/DNA complexes prepared from the 56 water-soluble/DNA-complexing polymers previously identified were prepared as described above at a ratio of 1:20 (w/w)) using a commercially available plasmid containing the firefly luciferase reporter gene (pCMV-Luc). An appropriate volume of each sample was added to the cells using a multichannel pipettor (e.g., 15 μL yielded 300 ng DNA/well; 30 μL yielded 600 ng DNA/well). Controls employing poly (ethylene imine) (PEI) and polylysine, prepared at DNA/polymer ratios of 1:1, were prepared in a similar manner and included with DNA and no-DNA controls. Controls employing Lipofectamine 2000 (Invitrogen Corp.) were performed at several concentrations (0.1, 0.2, 0.4, and 0.6 pL) as described in the technical manual for this product (http://lifetechnologies.com). Cells were incubated for 4 hours, after which the serum-free growth medium was removed and replaced with 100 μL of phenol-red-free growth medium. Cells were incubated for an additional period of time (typically varied between 36 to 60 hours) and luciferase expression was determined using a commercially available assay kit (Tropix, Inc., Bedford, Mass.). Luminescence was quantified in white, solid-bottom polypropylene 96-well plates using a 96-well bioluminescence plate reader. Luminescence was expressed in relative light units and was not normalized to total cell protein in this assay.

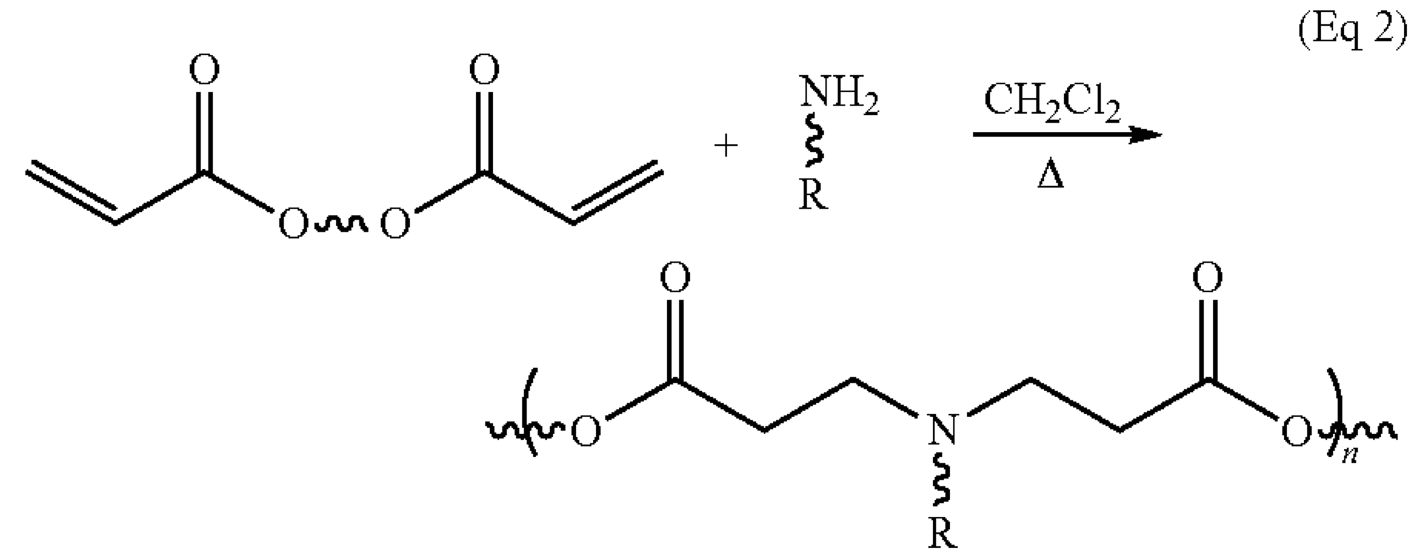
Results and Discussion

Poly(β-amino ester)s are hydrolytically degradable, condense plasmid DNA at physiological pH, and are readily synthesized via the conjugate addition of primary or secondary amines to diacrylates (Eq. 1 and 2) (Lynn et al. J. Am. Chem. Soc. 122:10761-10768, 2000; incorporated herein by reference). An initial screen of model polymers identified these materials as potential gene carriers and demonstrated that structural variations could have a significant impact on DNA binding and transfection efficacies (Lynn et al. J. Am. Chem. Soc. 122:10761-10768, 2000; incorporated herein by reference). We reasoned that this approach provided an attractive framework for the elaboration of large libraries of structurally-unique polymers for several reasons: 1) diamine and diacrylate monomers are inexpensive, commercially available starting materials, 2) polymerization can be accomplished directly in a single synthetic step, and 3) purification steps are generally unnecessary as no byproducts are generated during polymerization.

(Eq 1)



(Eq 2)



The paucity of commercially available bis(secondary amines) limits the degree of structural diversity that can be achieved using the above synthetic approach. However, the pool of useful, commercially available monomers is significantly expanded when primary amines are considered as potential library building blocks. Because the conjugate addition of amines to acrylate groups is generally tolerant of functionalities such as alcohols, ethers, and tertiary amines (Ferruti et al. Adv. Polym. Sci. 58:55-92, 1984; incorporated herein by reference), we believed that the incorporation of functionalized primary amine monomers into our synthetic strategy would serve to broaden structural diversity. Diacrylate monomers A-G and amine monomers 1-20 were selected for the synthesis of an initial screening library.

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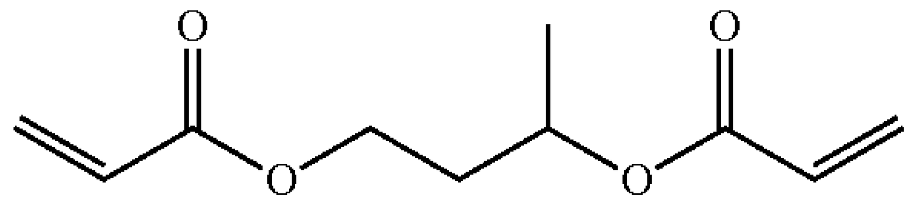

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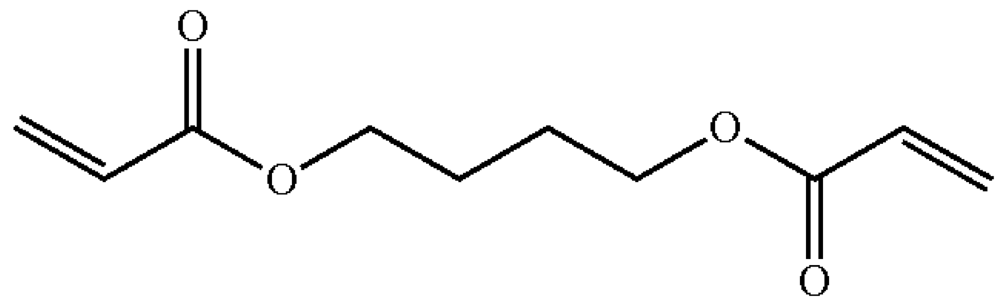

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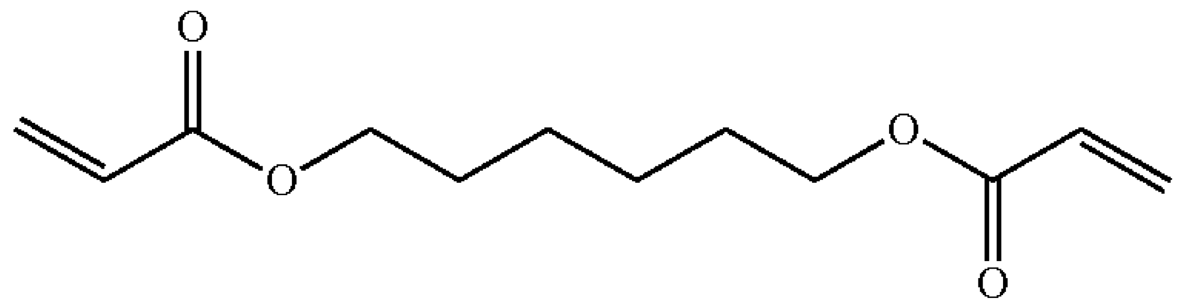

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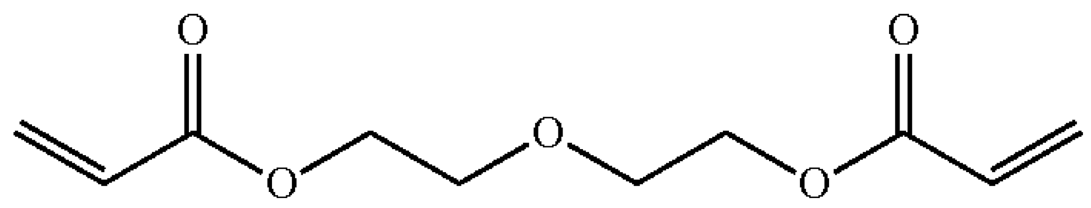

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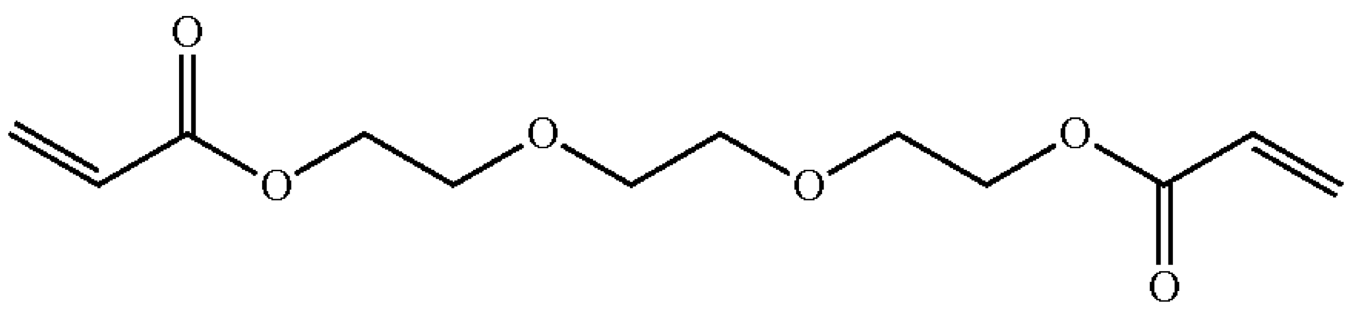

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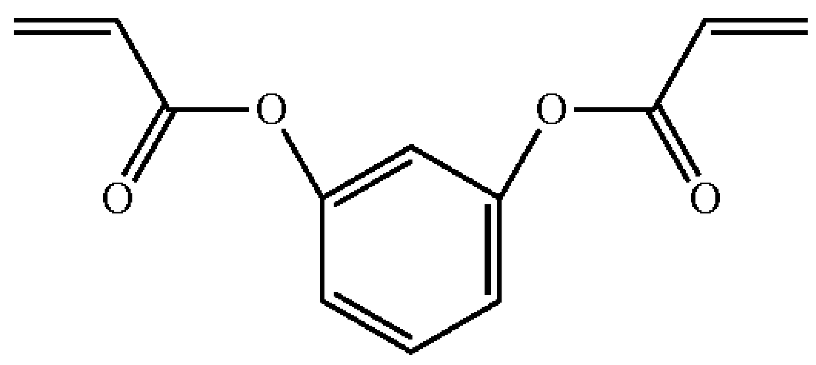

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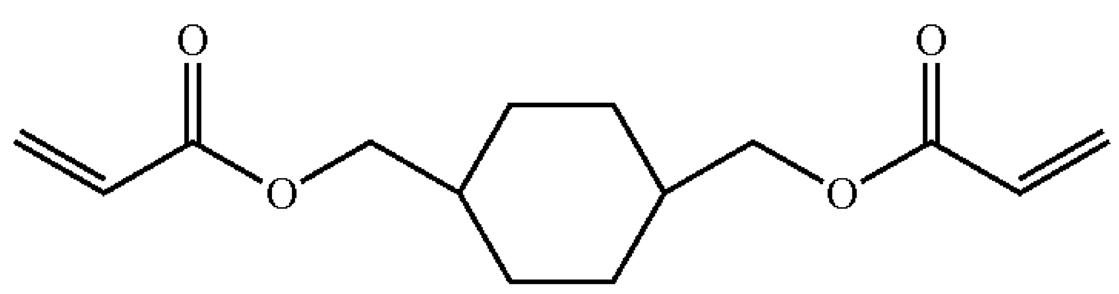

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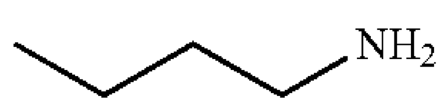

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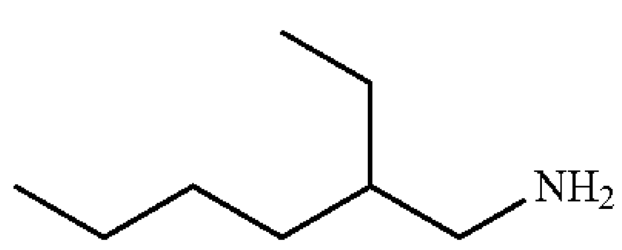

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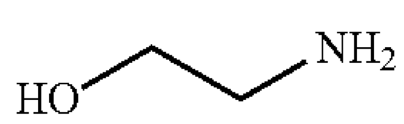

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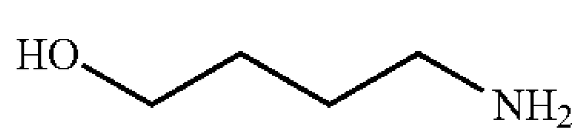

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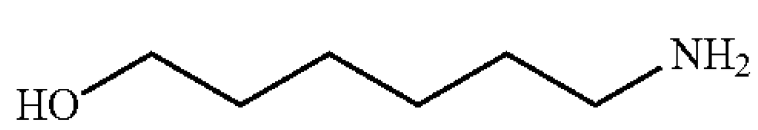

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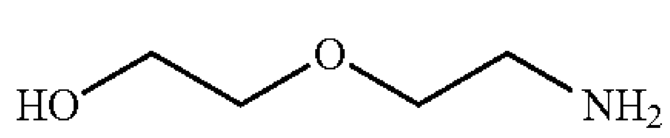

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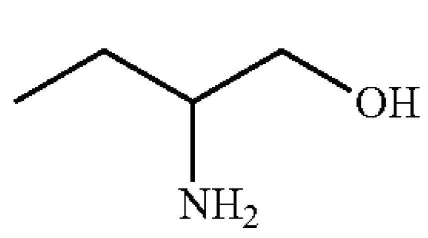

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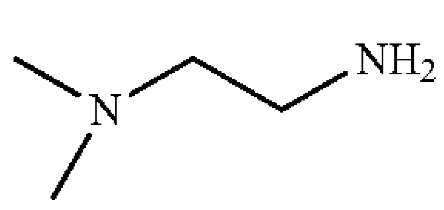

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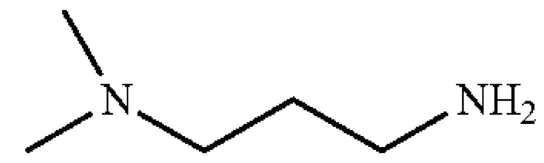

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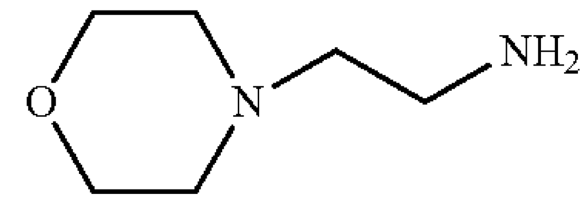

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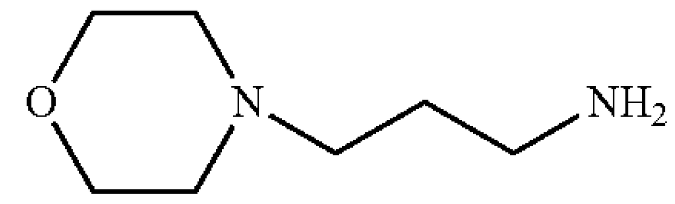

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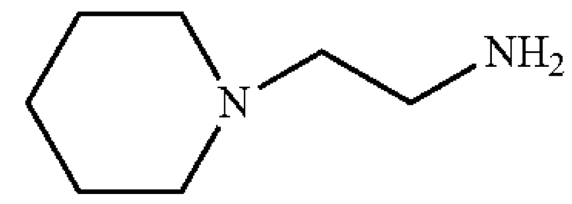

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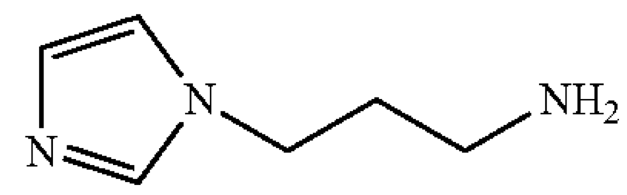

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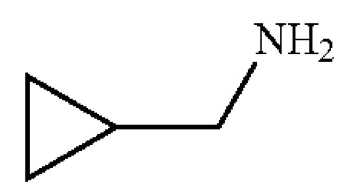

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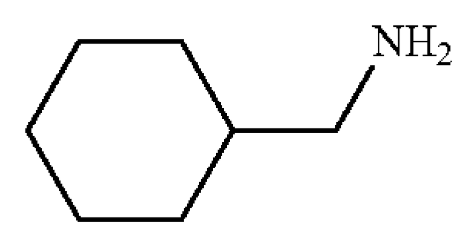

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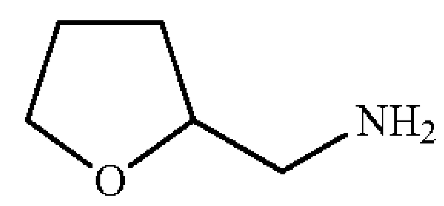

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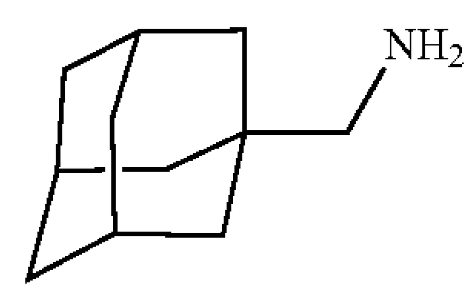

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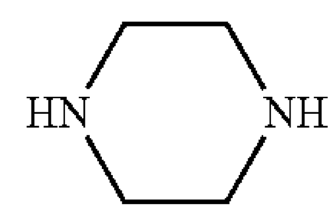

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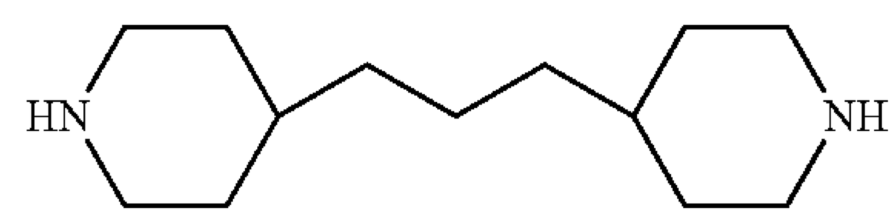


The size of the library constructed from this set of monomers (7 diacrylates×20 amines=140 structurally-unique polymers) was chosen to be large enough to incorporate sufficient diversity, yet small enough to be practical without the need for automation in our initial studies. It was unclear at the outset whether a polymer such as G16 (formed from hydrophobic and sterically bulky monomers G and 16) would be water-soluble at physiological pH or be able to condense DNA sufficiently. However, monomers of this type were deliberately incorporated to fully explore diversity space, and in anticipation that this library may ultimately be useful as a screening population for the discovery of materials for applications other than gene delivery (For a report on the parallel synthesis and screening of degradable polymers for tissue engineering, see: Brocchini et al. J. Am. Chem. Soc. 119: 4553-4554, 1997, incorporated herein by reference; Lynn et al. Angew. Chem. Int. Ed. 40:1707-1710, 2001; incorporated herein by reference).

Polymerization reactions were conducted simultaneously as an array of individually labeled vials. Reactions were performed in methylene chloride at 45° C. for 5 days, and polymers were isolated by removal of solvent to yield 600-800 mg of each material. Reactions performed on this scale provided amounts of each material sufficient for routine analysis by GPC and all subsequent DNA-binding, toxicity, and transfection assays. A survey of 55% of the library by GPC indicated molecular weights ranging from 2000 to 50 000 (relative to polystyrene standards). As high molecular weights are not required for DNA-complexation and transfection (as shown below) (Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; incorporated herein by reference), this library provided a collection of polymers and oligomers suitable for subsequent screening assays.

Of the 140 members of the screening library, 70 samples were sufficiently water-soluble (2 mg/mL, 25 mM acetate buffer, pH=5.0) to be included in an electrophoretic DNA-binding assay (FIG. **9**). To perform this assay as rapidly and efficiently as possible, samples were mixed with plasmid DNA at ratios of 1:5 and 1:20 (DNA/polymer, w/w) in 96-well plates and loaded into an agarose gel slab capable of assaying up to 500 samples using a multi-channel pipettor. All 70 water-soluble polymer samples were assayed simultaneously at two different DNA/polymer ratios in less than 30 minutes. As shown in FIG. **9**, 56 of the 70 water-soluble polymer samples interacted sufficiently with DNA to retard migration through the gel matrix (e.g., A4 or A5), employing the 1:20 DNA/polymer ratio as an exclusionary criterion. Fourteen polymers were discarded from further consideration (e.g., A7 and A8), as these polymers did not complex DNA sufficiently.

The DNA-complexing materials identified in the above assay were further investigated in transfection assays employing plasmid DNA and the COS-7 cell line. All assays were performed simultaneously with the firefly luciferase reporter gene (pCMV-Luc) and levels of expressed protein were determined using a commercially available assay kit and a 96-well luminescence plate reader. FIG. **10** displays data generated from an assay employing pCMV-Luc (600 ng/well) at DNA/poly ratios of 1:20 (w/w). The majority of the polymers screened did not mediate transfection above a level typical of "naked" DNA (no polymer) controls under these conditions. However, several wells expressed higher levels of protein and the corresponding polymers were identified as "hits" in this assay. Polymers B14 ($M_w$=3180) and G5 ($M_w$=9170), for example, yielded transfection levels 4-8 times higher than control experiments employing poly(ethylene imine) (PEI), a polymer conventionally employed as a synthetic transfection vector (Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; incorporated herein by reference), and transfection levels within or exceeding the range of expressed protein using Lipofectamine 2000 (available from Invitrogen Corp. (Carlsbad, Calif.)), a leading commercially available lipid-based transfection vector system. Polymers A14, C5, G7, G10, and G12 were also identified as positive "hits" in the above experiment, but levels of gene expression were lower than B14 and G5.

Structural differences among synthetic polymers typically preclude a general set of optimal transfection conditions. For example, polymers C5, C14, and G14 were toxic at the higher concentrations employed above (Determined by the absence of cells in wells containing these polymers as observed upon visual inspection. These polymers were less toxic and mediated transfection at lower concentration.), but mediated transfection at lower DNA and polymer concentrations (data not shown). The assay system described above can easily be modified to evaluate polymers as a function of DNA concentration, DNA/polymer ratio, cell seeding densities, or incubation times. Additional investigation will be required to more fully evaluate the potential of this screening library, and experiments to this end are currently underway.

The assays above were performed in the absence of chloroquine, a weak base commonly added to enhance in vitro transfection (Putnam et al. Proc. Natl. Acad. Sci. USA 98:1200-1205, 2001; Benns et al. Bioconjugate Chem. 11:637-645, 2000; Midoux et al. Bioconjugate Chem. 10:406-411, 1999; Kabanov et al., in Self-Assembling Complexes for Gene Delivery: From Laboratory to Clinical Trial, John Wiley and Sons, New York, 1998; each of which is incorporated herein by reference), and the results therefore reflect the intrinsic abilities of those materials to mediate transfection. The polymers containing monomer 14 are structurally similar to other histidine containing "proton sponge" polymers (Putnam et al. Proc. Natl. Acad. Sci. USA 98:1200-1205, 2001; Benns et al. Bioconjugate Chem. 11:637-645, 2000; Midoux et al. Bioconjugate Chem. 10:406-411, 1999; each of which is incorporated herein by reference), and could enhance transfection by buffering acidic intracellular compartments and mediating endosomal escape (Putnam et al. Proc. Natl. Acad. Sci. USA 98:1200-1205, 2001; Benns et al. Bioconjugate Chem. 11:637-645, 2000; Midoux et al. Bioconjugate Chem. 10:406-411, 1999; Boussif et al. Proc. Natl. Acad. Sci. USA 92:7297-7301, 1995; each of which is incorporated herein by reference). The efficacy of polymers containing monomer 5 is surprising in this context, as these materials do not incorporate an obvious means of facilitating endosomal escape. While the efficacy of these latter polymers is not yet understood, their discovery helps validate our parallel approach and highlights the value of incorporating structural diversity, as these polymers may not have been discovered on an ad hoc basis. Polymers incorporating hydrophilic diacrylates D and E have not produced "hits" under any conditions thus far, providing a possible basis for the development of more focused libraries useful for the elucidation of structure/activity relationships.

We have generated a library of 140 degradable polymers and oligomers useful for the discovery of new DNA-complexing materials and gene delivery vectors. Several of these materials are capable of condensing DNA into structures small enough to be internalized by cells and release the DNA in a transcriptionally active form. The total time currently required for library design, synthesis, and initial screening assays is approximately two weeks. However, the incorporation of robotics and additional monomers could significantly accelerate the pace at which new DNA-complexing materials and competent transfection vectors are identified.

## Example 4

### Semi-Automated Synthesis and Screening of a Large Library of Degradable Cationic Polymers for Gene Delivery

One of the major barriers to the success of gene therapy in the clinic is the lack of safe and efficient methods of delivering nucleic acids. Currently, the majority of clinical trials use modified viruses as delivery vehicles, which, while effective at transferring DNA to cells, suffer from potentially serious toxicity and production problems (Somia et al. Nat Rev Genet. 1:91 (2000); incorporated herein by reference). In contrast, non-viral systems offer a number of potential advantages, including ease of production, stability, low immunogenicity and toxicity, and reduced vector size limitations (Ledley Human Gene Therapy 6:1129 (1995); incorporated herein by reference). Despite these advantages, however, existing non-viral delivery systems are far less efficient than viral vectors (Luo et al. Nature Biotechnology 18:33 (2000); incorporated herein by reference).

One promising group of non-viral delivery compounds are cationic polymers, which spontaneously bind and condense DNA. A wide variety of cationic polymers that transfect in vitro have been characterized, both natural, such as protein (Fominaya et al. Journal of Biological Chemistry 271:10560 (1996); incorporated herein by reference) and peptide systems (Schwartz et al. Curr Opin Mol Ther. 2:162 (2000); incorporated herein by reference), and synthetic polymers such as poly(ethylene imine) (Boussif et al. Proceedings of the National Academy of Sciences of the United States of America 92:7297 (1995);incorporated herein by reference) and dendrimers (Kabanov et al. Self-assembling complexes for gene delivery: from laboratory to clinical trial, Wiley, Chichester; N.Y., 1998; incorporated herein by reference). Recent advances in polymeric gene delivery have in part focused on making the polymers more biodegradable to decrease toxicity. Typically, these polymers contain both chargeable amino groups, to allow for ionic interactions with the negatively charged phosphate backbone of nucleic acids, and a biodegradable linkage such as a hydrolyzable ester linkage. Several examples of these include poly(alpha-(4-aminobutyl)-L-glycolic acid) (Lim et al. Journal of the American Chemical Society 122:6524 (2000); incorporated herein by reference), network poly(amino ester) (Lim et al. Bioconjugate Chemistry 13:952 (2002); incorporated herein by reference), and poly(beta-amino ester)s (Lynn et al. Journal of the American Chemical Society 122:10761 (2000); Lynn et al. Journal of the American Chemical Society 123: 8155 (2001); each of which is incorporated herein by reference). Poly(beta-amino ester)s are particularly interesting because they show low cytotoxicity and are easily synthesized via the conjugate addtion of a primary amine or bis (secondary amine) to a diacrylate (FIG. **11**) (Lynn et al. Journal of the American Chemical Society 122:10761 (2000); Lynn et al. Journal of the American Chemical Society 123: 8155 (2001); each of which is incorporated herein by reference).

Traditional development of new biomedical polymers has been an iterative process. Polymers were typically designed one at a time and then individually tested for their properties. More recently, attention has focused on the development of parallel, combinatorial approaches that facilitate the generation of structurally-diverse libraries of polymeric biomaterials (Brocchini Advanced Drug Delivery Reviews 53:123 (2001); incorporated herein by reference). This combinatorial approach has also been applied to the discovery of gene delivery polymers. For example, Murphy et al. generated a targeted combinatorial library of 67 peptoids via solid-phase synthesis and screened them to identify new gene delivery agents (Murphy et al. Proceedings of the National Academy of Sciences of the United States of America 95:1517 (1998); incorporated herein by reference).

In this Example is described new tools for high-throughput, parallel combinatorial synthesis and cell-based screening of a large library of 2350 structurally diverse, poly(beta-amino ester)s. This approach allows for the screening of polymers in cell-based assays without the polymers ever leaving the solution phase following synthesis. This approach combined with the use of robotic fluid handling systems allows for the generation and testing of thousands of synthetic polymers in cell-based assays in a relatively short amount of time. Using this approach, 46 new polymers that perform as well or better than conventional non-viral delivery systems such as poly(ethylene imine) have been identified.

Results and Discussion

High-throughput polymer synthesis. The primary factors limiting the throughput and automation of poly(beta-amino esters) synthesis and testing was the viscosity of monomer and polymer solutions, and the difficulty with manipulating the solid polymer products. While automation of liquid handling is straightforward using conventional robotics, the manipulation of solids and viscous liquids on a small scale is not. Therefore, a system was developed in which polymers could be synthesized and screened in cell-based assays without leaving the solution phase. Since this would require the presence of residual solvent in the cell assays, the relatively non-toxic solvent, dimethyl sulfoxide (DMSO) was chosen. DMSO is a commonly used solvent in cell culture and is routinely used in storing frozen stocks of cells. DMSO is miscible with water and is generally well tolerated in living systems.

The first step in preparing for high-throughput synthesis was to identify conditions that would allow for the production of polymer yet possess a manageable viscosity. Small scale, pilot experiments showed that polymerization could be performed effectively at 1.6 M in DMSO at 56° C. for 5 days. Based on these experiments, a general strategy was developed for polymer synthesis and testing. All monomers (FIG. **12**) were diluted to 1.6 M in DMSO, and then using both a fluid-handling robot and a 12 channel micropipettor, we added 150 microliters of each amine and diacrylate monomer into a polypropylene deep well plate and then sealed it with aluminum foil. The plates were placed on an orbital shaker and incubated at 56° C. for 5 days. To compensate for the increased viscosity of polymeric solutions, 1 ml of DMSO was added to each well of the plate, and the plates were then stored at 4° C. until further use. These methods allos for 2350 reactions in a single day. Furthermore, the production and storage of polymers in a 96-well format allowed for an easy transition into 96-well format cell-based testing of polymer transfection efficiency.

High-throughput polymer testing. Once synthesized, all polymers were tested for their ability to deliver the Luciferase expressing plasmid, pCMV-luc, into the monkey kidney fibroblast cell line COS-7. Due to the large size of the polymer library, a high-throughput method for cell based screening of transfection efficience was developed. Since polymers were stored in 96 well plates, all polymer dilutions, DNA complexation, and cell transfections were performed in parallel by directly transferring polymers from plate to plate using a liquid handling robot. All polymers were synthesized using the same concentration of amine monomer, thus comparison between polymers at a fixed amine monomer:DNA phosphate ratio (N:P ratio) was straightforward. While the amine monomers contain either one, two, or three amines per monomer, initial broad-based screens for transfection efficiency were greatly simplified by maintaining a constant monomer concentration in all wells of a single plate, and therefore a constant volume of polymer solution per reaction (see methods below).

The efficiency of in vitro transfection using cationic polymers such as poly(ethylene imine) is very sensitive to the ratio of polymer to DNA present during complex formation (Gebhart et al. Journal of Controlled Release 73:401 (2001); incorporated herein by reference). N:P rations of 10:1, 20:1, and 40:1 were selected for our initial screens based on previous experience with these types of polymers. Using our high-throughput system, we screened all 2350 polymers at these three ratios. Transfection values at the best condition for each polymer were tabulated into a histogram (FIG. **13**). These results were compared to three controls: naked DNA (no transfection agent), poly(ethylene imine) (PEI), and Lipofectamine 2000. The low, residual levels of DMSO present in the transfection solutions did not affect transfection efficiency of either naked DNA or PEI. Thirty-three of the 2350 polymers were found to be as good or better than PEI in this unoptimized system.

Since cationic polymer transfections tend to be sensitive to polymer:DNA ratio, we decided to optimize transfection conditions with the best polymers from our preliminary screen. Using the results above as a rough guide, the transfection condition for the best 93 polymers were optimized by testing them at N:P ratios above and below the optimal transfection conditions identified in the broad based screen. In order to develop a high-throughput optimization system, these were tested using an N:P ratio multiplier system to simplify simultaneous testing and plate-to-plate transfer. Starting with the best N:P ratio identified in the preliminary screen, the polymers were retested at six N:P ratios equal to the best ratio times 0.5, 0.75, 1.0, 1.25, 1.5, and 1.75, in triplicate. For example, if the optimal ratio identified in the screen for a given polymer was 20: 1, then that polymer was rescreened in triplicate at N:P ratios of 10:1, 15:1, 20:1, 25:1, 30:1, and 35:1. The average transfection efficiencies with standard deviation from the best condition for the best 50 polymers are shown in FIG. **14**, along with control data. In this experiment, 46 polymers were identified that transfect as good or better than PEI. All 93 of these polymers were also tested for their ability to bind DNA using agarose gel electrophoresis (FIG. **15**). Interestingly, while almost all of the polymers bind DNA as expected, two polymers that transfect at high levels do not: M17 and KK89 (FIG. **14**).

To further examine the transfection properties of these polymers, ten high transfecting polymers were tested for their ability to deliver the green fluorescent protein plasmid, pCMV-eGFP. Unlike pCMV-luc, pCMV-eGFP provides information concerning what percentage of cells is transfected. High levels of transfection were observed for all 10 polymers, and two of the best are shown in FIG. **16**.

The "hits" identified in the above assays reveal a surprisingly diverse and unexpected collection of polymers. Particularly surpising is the large collection of hydrophobic, D-monomer-based polymers. In fact, the diacrylate monomers used to make the best performing 50 polymers are almost always hydrophobic. Further analysis reveals two more common features of the effective polymers: 1) twelve of the 26 polymers that are better than the best conventional reagent, Lipofectamine 2000, have mono- an di-alcohol side groups, and 2) linear, bis(secondary amines) are also prevelant in the hit structures. Also surprising was the identification of two polymers that transfect at high levels but do not appear to bind DNA (KK89 and M17). Both are also insoluble at pH 5 and pH 7. Their ability to facilitate DNA uptake may be due to permeabilization of the cellular membrane.

Also important for the function of gene delivery polymers is length (Remy et al. Advanced Drug Delivery Reviews 30:85 (1998); Schaffer et al. Biotechnol Bioeng 67:598 (2000); each of which is incorporated herein by reference). Using these results as a framework, a range of polymer lengths for each hit polymer may be prepared by carefully varying relative monomer concentrations. Evidence shows that (1) like PEI, poly(beta-amino ester) length is important in the gene delivery proficiency of these polymers, and (2) that the hits identified here can be resynthesized using conventional methods and still deliver DNA effectively.

Experimental Protocols

Polymer synthesis. Monomers were purchased from Aldrich (Milwaukee, Wis.), TCI (Portland, Oreg.), Pfaltz & Bauer (Waterbury, Conn.), Matrix Scientific (Columbia, S.C.), Acros-Fisher (Pittsburg, Pa.), Scientific Polymer (Ontario, N.Y.), Polysciences (Warrington, Pa.), and Dajac monomer-polymer (Feasterville, Pa.). These were dissolved in DMSO (Aldrich) to a final concentration of 1.6 M. All possible pair wise combinations amine and diacrylate monomers were added in 150 μl aliquots to each well of 2 ml 96 well polypropylene masterblock deep well plates (Griener America, Longwood, Fla.). The plates were sealed with aluminum foil, and incubated at 56° C. while rotating on an orbital shaker. After 5 days, 1 ml of DMSO was added to each well, and the plates were resealed and stored frozen at 4° C. until ready to be used. **0**Transfection experiments. 14,000 cos-7 cells (ATCC, Manassas, Va.) were seeded into each well of a solid white or clear 96 well plate (Corning-Costar, Kennebunk, Me.) and allowed to attached overnight in growth medium, composed of: 500 ml phenol red minus DMEM, 50 ml heat inactivated FBS, 5 ml penicillin/streptomycin (Invitrogen, Carlsbad, Calif.). Each well of a master block 96-well plate was filled with 1 ml of 25 mM sodium acetate pH 5. To this, 40 μl, 20 μl, or 10 μl of the polymer/DMSO solution was added. 25 μl of the diluted polymer was added to 25 μl of 60 μg/ml pCMV-luc DNA (Promega, Madison, Wis.) or pEGFP-N1 (Invitrogen) in a half volume 96 well plate. These were incubated for 10 minutes, and then 30 μl of the polymer-DNA solution was added to 200 μl of Optimem with sodium bicarbonate (Invitrogen) in 96 well polystyrene plates. The medium was removed from the cells using a 12-channel wand (V & P Scientific, San Diego, Calif.) after which 150 μl of the optimem-polymer-DNA solution was immediately added. Complexes were incubated with the cells for 1 hour and then removed using the 12-channel wand and replaced with 105 μl of growth medium. Cells were allowed to grow for three days at 37° C., 5% $CO_2$ and then analyzed for luminescence (pCMV-luc) or fluorescence (pEGFP-N1). Control experiments were performed by as described above, but using poly (ethylene imine) MW 25,000 (Aldrich) replacing synthesized polymer, and at polymer:DNA weight ratios of 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1. Lipofectamine 2000 (Invitrogen) transfections were performed as described by the vendor, except that complexes were removed after 1 hour.

Luminescence was analyzed using bright-glo assay kits (Promega). Briefly, 100 μl of bright-glo solution was added to each well of the microtiter plate containing media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, Tenn.). In some cases, a neutral density filter (Chroma, Brattleboro, Vt.) was used to prevent saturation of the luminometer. A standard curve for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth media in white microtiter plates. eGFP expression was examined using a Zeiss Aciovert 200 inverted microscope.

Agarose gel electrophoresis DNA-binding assays were done at a N:P ratio of 40: 1, as previously described (Lynn et al. Journal of the American Chemical Society 123:8155 (2001): incorporated herein by reference). All liquid handling was performed using an EDR384S/96S robot (Labcyte, Union City, Calif.) or a 12 channel micropippettor (Thermo Labsystems, Vantaa, Finland) in a laminar flow hood.

Example 5

Synthesis of Poly(beta-amino esters) Optimized for Highly Effective Gene Delivery

The effect of molecular weight, polymer/DNA ratio, and chain end-group on the transfection properties of two unique poly(β-amino ester) structures was determined. These factors can have a dramatic effect on gene delivery function. Using high throughput screening methods, poly(β-amino esters) that transfect better than PEI and Lipofectamine 2000 (two of the best commercially available transfection reagents) have been discovered.

Materials and Methods

Polymer Synthesis. Poly-1 and Poly-2 polymers were synthesized by adding 1,4-butanediol diacrylate (99+%) and 1,6-hexanediol diacrylate (99%), respectively, to 1-amino butanol (98%). These monomers were purchased from Alfa Aesar (Ward Hill, Mass.). Twelve versions each of Poly-1 and Poly-2 were generated by varying the amine/diacrylate stoichiometric ratio. To synthesize each of the 24 unique polymers, 400 mg of 1-amino butanol was weighed into an 8 mL sample vial with Telfon-lined screw cap. Next, the appropriate amount of diacrylate was added to the vial to yield a stoichiometric ratio between 1.4 and 0.6. A small Telfon-coated stir bar was then put in each vial. The vials were capped tightly and placed on a multi-position magnetic stirplate residing in an oven maintained at 100° C. After a reaction time of 5 hr, the vials were removed from the oven and stored at 4° C. All polymers were analyzed by GPC.

Gel Permeation Chromatography (GPC). GPC was performed using a Hewlett Packard 1100 Series isocratic pump, a Rheodyne Model 7125 injector with a 100-μL injection loop, and a Phenogel MXL column (5μ mixed, 300×7.5 mm, Phenomenex, Torrance, Calif.). 70% THF/30% DMSO (v/v)+0.1 M piperidine was used as the eluent at a flow rate of 1.0 mL/min. Data was collected using an Optilab DSP interferometric refractometer (Wyatt Technology, Santa Barbara, Calif.) and processed using the TriSEC GPC software package (Viscotek Corporation, Houston, Tex.). The molecular weights and polydispersities of the polymers were determined relative to monodisperse polystyrene standards.

Luciferase Transfection Assays. COS-7 cells (ATCC, Manassas, Va.) were seeded (14,000 cells/well) into each well of an opaque white 96-well plate (Corning-Costar, Kennebunk, Me.) and allowed to attach overnight in growth medium. Growth medium was composed of 90% phenol red-free DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, Calif.). To facilitate handling, polymers stock solutions (100 mg/mL) were prepared in DMSO solvent. A small residual amount of DMSO in the transfection mixture does not affect transfection efficiency and does not result in any observable cytotoxicity. Working dilutions of each polymer were prepared (at concentrations necessary to yield the different polymer/DNA weight ratios) in 25 mM sodium acetate buffer (pH 5). 25 μl of the diluted polymer was added to 25 μl of 60 μg/ml pCMV-Luc DNA (Elim Biopharmaceuticals, South San Francisco, Calif.) in a well of a 96-well plate. The mixtures were incubated for 10 minutes to allow for complex formation, and then 30 μl of the each of the polymer-DNA solutions were added to 200 μl of Opti-MEM with sodium bicarbonate (Invitrogen) in 96-well polystyrene plates. The growth medium was removed from the cells using a 12-channel aspirating wand (V&P Scientific, San Diego, Calif.) after which 150 μl of the Opti-MEM-polymer-DNA solution was immediately added. Complexes were incubated with the cells for 1 hr and then removed using the 12-channel wand and replaced with 105 μl of growth medium. Cells were allowed to grow for three days at 37° C., 5% $CO_2$ and were then analyzed for luciferase expression. Control experiments were also performed with PEI (MW=25,000, Sigma-Aldrich) and Lipofectamine 2000 (Invitrogen). PEI transfections were performed as described above, but using polymer:DNA weight ratios of 1:1. Lipofectamine 2000 transfections were performed as described by the vendor, except that complexes were removed after 1 hour.

Luciferase expression was analyzed using Bright-Glo assay kits (Promega). Briefly, 100 μl of Bright-Glo solution was added to each well of the 96-well plate containing media and cells. Luminescence was measured using a Mithras Luminometer (Berthold, Oak Ridge, Tenn.). A 1% neutral density filter (Chroma, Brattleboro, Vt.) was used to prevent saturation of the luminometer. A standard curve for Luciferase was generated by titration of Luciferase enzyme (Promega) into growth media in an opaque white 96-well plate.

Measurement of Cytotoxicity. Cytotoxicity assays were performed in the same manner as the luciferase transfection experiments with the following exception. Instead of assaying for luciferase expression after 3 days, cells were assayed for metabolic activity using the MTT Cell Proliferation Assay kit (ATCC) after 1 day. 10 μL of MTT Reagent was added to each well. After 2 hr incubation at 37° C., 100 μL of Detergent Reagent was added to each well. The plate was then left in the dark at room temperature for 4 hr. Optical absorbance was measured at 570 nm using a SpectaMax 190 microplate reader (Molecular Devices, Sunnyvale, Calif.) and converted to % viability relative to control (untreated) cells.

Cellular Uptake Experiments. Uptake experiments were done as previously described, with the exception that a 12-well plate format was used instead of a 6-well plate format (Akinc, A., et al., Parallel synthesis and biophysical characterization of a degradable polymer library of gene Delivery. J. Am. Chem. Soc., 2003; incorporated herein by reference). COS-7 cells were seeded at a concentration of $1.5\times10^5$ cells/well and grown for 24 hours prior to performing the uptake experiments. Preparation of polymer/DNA complexes was done in the same manner as in the luciferase transfection experiments, the only differences being an increase in scale (2.5 μg DNA per well of 12-well plate as opposed to 600 ng DNA per well of 96-well plate) and the use of Cy5-labeled plasmid instead of pCMV-Luc (Akinc, A. and R. Langer, Measuring the pH environment of DNA delivered using nonviral vectors: Implications for lysosomal trafficking. Biotechnol. Bioeng., 2002. 78(5): p. 503-8; incorporated herein by reference). As in the transfection experiments, complexes were incubated with cells for 1 hr to allow for cellular uptake by endocytosis. The relative level of cellular uptake was quantified using a flow cytometer to measure the fluorescence of cells loaded with Cy5-labeled plasmid.

GFP Transfections. GFP transfections were carried in COS-7 (green monkey kidney), NIH 3T3 (murine fibroblast), HepG2 (human hepatocarcinoma), and CHO (Chinese Hamster Ovary) cell lines. All cell lines were obtained from ATCC (Manassas, Va.) and maintained in DMEM containing 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin at 37° C. in 5% $CO_2$ atmosphere. Cells were seeded on 6-well plates and grown to roughly 80-90% confluence prior to performing the transfection experiments. Polymer/DNA complexes were prepared as described above using the pEGFP-N1 plasmid (Clontech, Palo Alto, Calif.) (5 μg/well). Complexes were diluted in 1 mL Opti-MEM and added to the wells for 1 hr. The complexes were then removed and fresh growth media was added to the wells. After 2 days, cells were harvested and analyzed for GFP expression by flow cytometry. Propidium iodide staining was used to exclude dead cells from the analysis.

Flow Cytometry. Flow cytometry was performed with a FACSCalibur (Becton Dickinson) equipped with an argon ion laser capable of exciting GFP (488 nm excitation) and a red diode laser capable of exciting Cy5 (635 nm excitation). The emission of GFP was filtered using a 530 nm band pass filter and the emission of Cy5 was filtered using a 650 long pass filter. The cells were appropriately gated by forward and side scatter and 30,000 events per sample were collected.

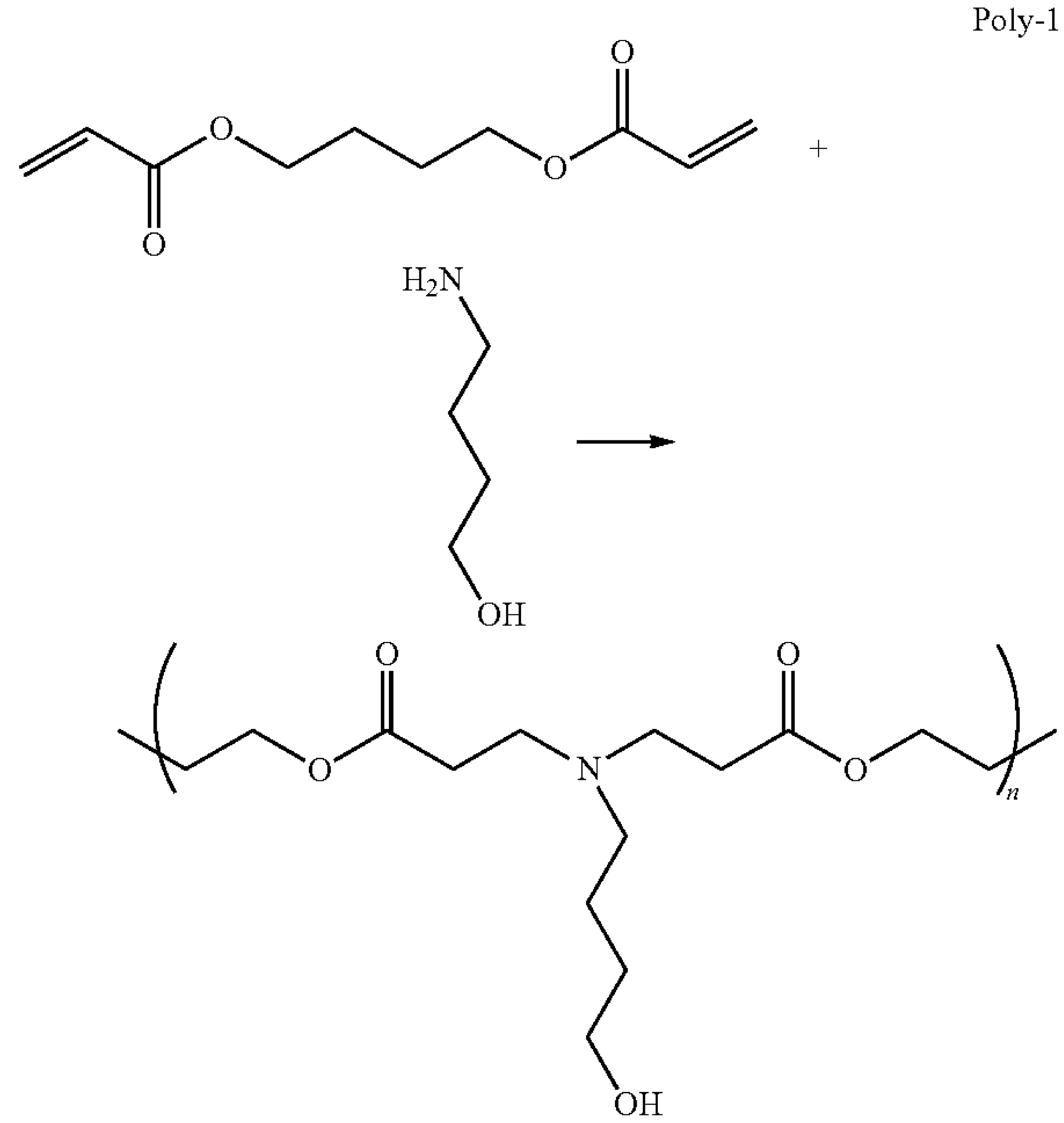
Results and Discussion

Polymer Synthesis. As previously described (Lynn, D. M. and R. Langer, Degradable poly (β-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. J. Am. Chem. Soc., 2000.122(44): p. 10761-10768; incorporated herein by reference), the synthesis of poly(β-amino esters) proceeds via the conjugate addition of amines to acrylate groups. Because the reaction is a step polymerization, a broad, statistical distribution of chain lengths is obtained, with average molecular weight and chain end-groups controlled by monomer stoichiometry (Flory, P., in Principles of Polymer Chemistry. 1953, Cornell University Press: Ithaca, N.Y. p. 40-46, 318-323; Odian, G., Step Polymerizaton, in Principles of Polymerization. 1991, John Wiley & Sons, Inc.: New York. p. 73-89; each of which is incorporated herein by reference). Molecular weight increases as the ratio of monomers nears stoichiometric equivalence, and an excess of amine or diacrylate monomer results in amine- or acrylate-terminated chains, respectively. For this class of polymers, precise control of stoichiometry is essential for controlling polymer molecular weight. While monomer stoichiometry is the most important factor affecting chain length, consideration should also be given to competing side reactions that can impact the molecular weight and structure of polymer products. In particular, intramolecular cyclization reactions, where an amine on one end of the growing polymer chain reacts with an acrylate on the other end, can limit obtained molecular weights (Odian, G., Step Polymerizaton, in Principles of Polymerization. 1991, John Wiley & Sons, Inc.: New York. p. 73-89; incorporated herein by reference). These cyclic chains may also have properties that differ from those of their linear counterparts.

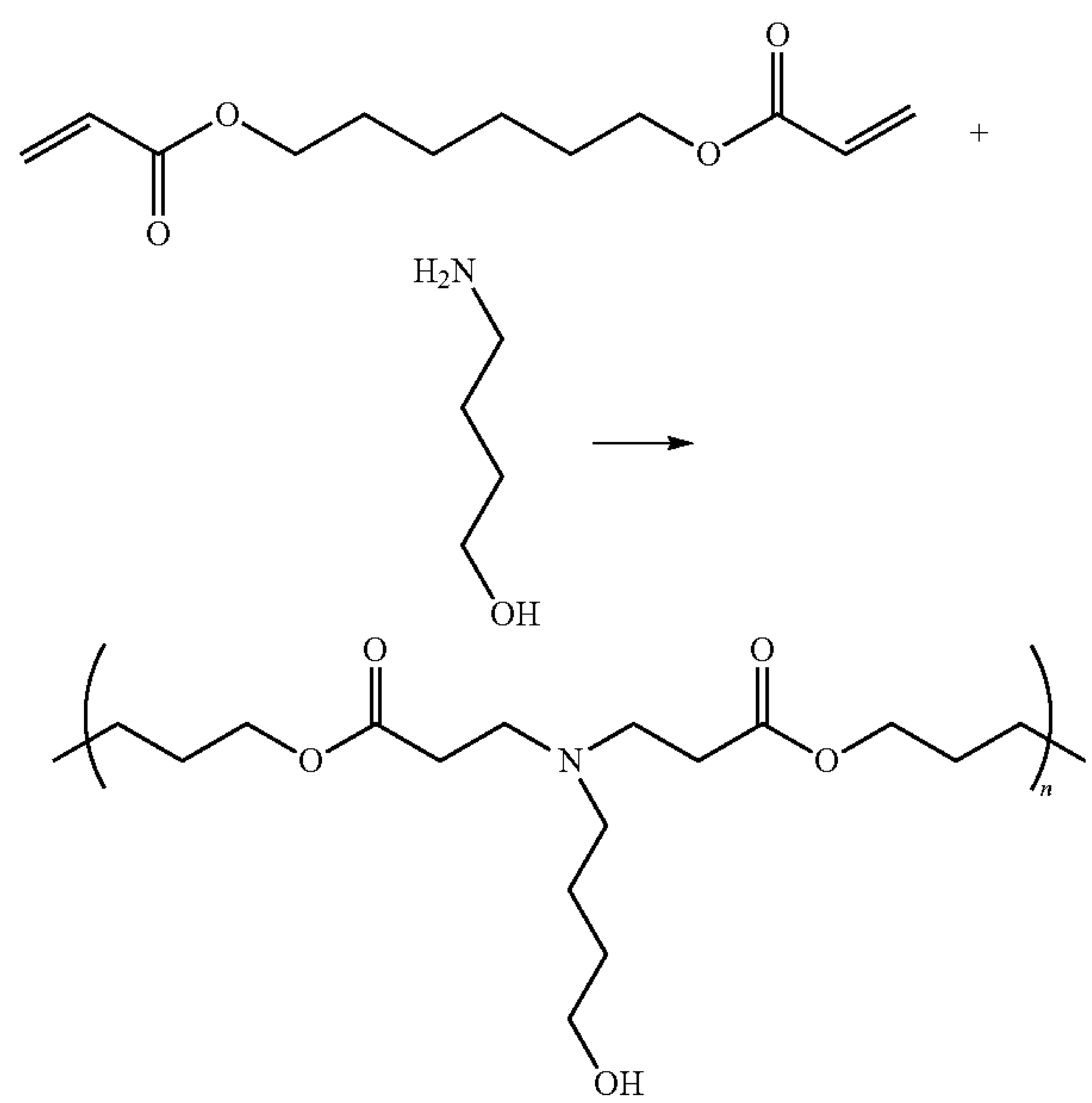
In this work, we have modified the previously reported polymerization procedure in order to better control monomer stoichiometry and to minimize cyclization reactions. First, the scale of synthesis was increased from roughly 0.5 g to 1 g to allow for control of stoichiometry within 1%. Further improvement in accuracy is limited by the purity (98-99%) of the commercially available monomers used. Second, all monomers were weighed into vials instead of being dispensed volumetrically. Discrepancies between actual and reported monomer densities were found to be non-negligible in some cases, leading to inaccuracies in dispensed mass. Third, polymerizations were performed in the absence of solvent to maximize monomer concentration, thus favoring the intermolecular addition reaction over the intramolecular cyclization reaction. Eliminating the solvent also provides the added benefits of increasing the reaction rate and obviating the solvent removal step. Finally, since the previously employed methylene chloride solvent was not used, the reaction temperature could be increased from 45° C. to 100° C. Increasing temperature resulted in an increased reaction rate and a decrease in the viscosity of the reacting mixture, helping to offset the higher viscosity of the solvent-free system. The combined effect of increased monomer concentration and reaction temperature resulted in a decrease in reaction time from roughly 5 days to 5 hours.

We synthesized polymers Poly-1 and Poly-2 by adding 1,4-butanediol diacrylate and 1,6-hexanediol diacrylate, respectively, to 1-amino butanol. Twelve unique versions of each polymer were synthesized by varying amine/diacrylate mole ratios between 0.6 and 1.4.

Poly-1



Poly-2



For both sets of polymers (Poly-1 and Poly-2), 7 of the 12 had amine/diacrylate ratios>1, resulting in amine-terminated polymers, and 5 of the 12 had amine/diacrylate ratios<1, resulting in acrylate-terminated polymers. After 5 hr reaction at 100° C., polymers were obtained as clear, slightly yellowish, viscous liquids. The polymers had observable differences in viscosity, corresponding to differences in molecular weight. Polymers were analyzed by organic phase gel permeation chromatography (GPC) employing 70% THF/30% DMSO (v/v)+0.1 M piperidine eluent. Polymer molecular weights ($M_w$) ranged from 3350 (Poly-1, amine/diacrylate=1.38) to 18,000 (Poly-1, amine/diacrylate=0.95), relative to polystyrene standards (FIG. **16**). Molecular weight distributions were monomodal with polydispersity indices (PDIs) ranging from 1.55 to 2.20.

Luciferase Transfection Results. Transfection experiments were performed with all 24 synthesized polymers (12 each of

Poly-1 and Poly-2) at 9 different polymer/DNA ratios to determine the impact of molecular weight, polymer/DNA ratio, and chain end-group on transfection efficiency (FIGS. **17** and **18**). As a model system, we used the COS-7 cell line and a plasmid coding for the firefly luciferase reporter gene (pCMV-Luc) (600 ng/well). To facilitate performance of the nearly 1000 transfections (data obtained in quadruplicate), experiments were done in 96-well plate format. Reporter protein expression levels were determined using a commercially available luciferase assay kit and a 96-well luminescence plate reader.

The data displayed in FIGS. **17** and **18** demonstrate that polymer molecular weight, polymer/DNA ratio, and chain end-group impact the transfection properties of both Poly-1 and Poly-2 polymers. One striking, and somewhat unexpected, result was that none of the acrylate-terminated polymers mediated appreciable levels of transfection under any of the evaluated conditions. This result may be more broadly applicable for poly(β-amino esters), as we have yet to synthesize a polymer, using an excess of diacrylate monomer, that mediates appreciable reporter gene expression at any of the polymer/DNA ratios we have employed. These findings suggest that perhaps only amine-terminated poly(β-amino esters) are suitable for use as gene delivery vehicles. In contrast, there were distinct regions of transfection activity in the MWpolymer/DNA space for amine-terminated versions of both Poly-1 and Poly-2 (FIGS. **17**-A and **18**-A). Maximal reporter gene expression levels of 60 ng luc/well and 26 ng luc/well were achieved using Poly-1 ($M_w$=13,100) and Poly-2 ($M_w$=13,400), respectively. These results compare quite favorably with PEI (polymer/DNA=1:1 w/w), which mediated an expression level of 6 ng luc/well (data not shown) under the same conditions.

While the highest levels of transfection occurred using the higher molecular weight versions of both polymer structures, the optimal polymer/DNA ratios for these polymers were markedly different (polymer/DNA=150 for Poly-1, polymer/DNA=30 for Poly-2). The transfection results we have obtained for Poly-1 and Poly-2 highlight the importance of optimizing polymer molecular weight and polymer/DNA ratio, and the importance of controlling chain end-groups. Further, the fact that two such similar polymer structures, differing by only two carbons in the repeat unit, have such different optimal transfection parameters emphasizes the need to perform these optimizations for each unique polymer structure. To improve our understanding of the obtained transfection results, we chose to study two important delivery characteristics that directly impact transgene expression, cytotoxicity and the ability to enter cells via endocytosis (Wiethoff, C. M. and C. R. Middaugh, Barriers to nonviral gene delivery. Journal of Pharmaceutical Sciences, 2003. 92(2): p. 203-217; incorporated herein by reference).

Cytotoxicity. We evaluated the cytotoxicities of the various polymer/DNA complexes using a standard MTT/thiazolyl blue dye reduction assay. The experiments were performed exactly as the transfection experiments described above, except that instead of assaying for reporter gene expression on day 3, the MTT assay was performed after day 1 (see Materials and Methods). We initially hypothesized that the lack of transfection activity observed for acrylate-terminated polymers may have been due to the cytotoxicity of the acrylate end-groups. FIGS. **19**-B and **20**-B do indicate that high concentrations of acrylate are cytotoxic, as viability is seen to decrease with increasing polymer/DNA ratio and decreasing polymer $M_w$ (lower $M_w$ corresponds to a higher concentration of end-groups). However, cytotoxicity of acrylates does not sufficiently explain the lack of transfection activity at lower polymer/DNA ratios or higher molecular weights. Data shown in FIG. **19**-A demonstrates that cytotoxicity is not a limiting factor for Poly-1 vectors, since cells remain viable even at the highest polymer/DNA ratios. On the other hand, the data displayed in FIG. **20**-A suggests that cytotoxicity is a major factor limiting the transfection efficiency of Poly-2 vectors, especially for the lower molecular weight polymers. This result may explain why transfection activity is non-existent or decreasing for Poly-2 vectors at polymer/DNA>30 (see FIG. **18**-A).

Cellular Uptake. The ability of polymer/DNA complexes to be taken up by cells was evaluated using a previously described flow cytometry-based technique to measure the fluorescence of vector-delivered DNA (Akinc, A., et al., Parallel synthesis and biophysical characterization of a degradable polymer library of gene delivery. J. Am. Chem. Soc., 2003; incorporated herein by reference). Briefly, polymer/DNA complexes were prepared using plasmid DNA covalently labeled with the fluorescent dye Cy5. To allow for comparison of the cellular uptake data with the gene expression data outlined above, complexes were formed at the same polymer/DNA ratios and in the same manner as in the transfection experiments. Labeled complexes were incubated with COS-7 cells for 1 hr at 37° C. to allow for uptake. The relative level of particle uptake was then quantified by measuring the fluorescence of cells loaded with Cy5-labeled DNA. The results of these uptake experiments are summarized in FIGS. **21** and **22**. Data shown in FIG. **21**-B and **22**-B suggest that the lack of transfection activity for the acrylate-terminated polymers is not due to cytotoxicity, as initially thought, but rather to an inability to enter the cell. Similarly, Poly-1 complexes are severely uptake-limited at all but the highest polymer/DNA ratios (FIG. **21**-A). While this data doesn't correlate exactly with the transfection results obtained for Poly-1, it is consistent with the fact that transfection activity is not observed until very high polymer/DNA ratios are employed. Poly-2 complexes show no appreciable cellular uptake at polymer/DNA ratios<30 and increasing levels of uptake as polymer/DNA ratios increase beyond 30 (FIG. **22**-A). This result, combined with the above cytotoxicity results, helps to explain the transfection activity of Poly-2 complexes. At polymer/DNA ratios less than 30, complexes do not effectively enter the cell, but as polymer/DNA ratios increase much beyond 30, cytotoxicity begins to limit transfection efficiency, resulting in optimal transfection activity near polymer/DNA=30.

Where endocytosis is the main route of cellular entry, the effective formation of nanometer-scale polymer/DNA complexes is one requirement for achieving high levels of cellular uptake (De Smedt, S. C., J. Demeester, and W. E. Hennink, Cationic polymer based gene delivery systems. Pharmaceutical Research, 2000. 17(2): p. 113-126; Prabha, S., et al., Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. International Journal of Pharmaceutics, 2002. 244(1-2): p. 105-115; each of which is incorporated herein by reference). The poor uptake levels observed for many of the polymer/DNA complexes may have been attributable to the unsuccessful formation of stable, nanoscale complexes. Unfortunately, the poor neutral pH solubility of the polymers prevented making dynamic light scattering measurements of complex size using the transfection medium (Opti-MEM reduced serum media, pH 7.2) as the diluent. The obtained readings were attributable to polymer precipitates, which were in some cases visible as turbidity in solutions of polymer in Opti-MEM. However, we were able to measure the effective diameters of complexes using 25 mM sodium acetate (pH 5) as the sample diluent. While this data cannot shed light on the size or stability of complexes in the transfection medium, it can indicate whether complexes were successfully formed prior to addition to the transfection medium. Specifically, we found that the lower molecular weight versions ($M_w$<10,700) of Poly-1 were unable to form nanoscale complexes, even in acetate buffer. In all other cases we found that nanometer-sized polymer/DNA complexes were formed. While these results may explain the poor uptake levels associated with low molecular weight versions of Poly-1, they do not satisfactorily explain the low uptake activity of the acrylate-terminated polymers or the dependence of polymer/DNA ratio on cellular uptake. Although particle size and stability are important factors impacting cellular uptake, it is likely that other, yet unidentified, factors must also be considered in order to provide a more complete explanation of the obtained cellular uptake results.

Enhancement of Transfection Using a Co-Complexing Agent. Both Poly-1 and Poly-2 require relatively high polymer/DNA weight ratios to achieve high levels of gene transfer. One explanation may be that, compared to other polymers often used to compact DNA (e.g., polylysine (PLL) and PEI), these polymers have relatively low nitrogen densities. Poly-1 has a molecular weight per nitrogen atom (MW/N) of 301, and Poly-2 has a MW/N of 329. By comparison, for PLL and PEI, these figures are roughly 65 and 43, respectively. It might be possible to reduce the amount of Poly-1 or Poly-2 necessary to achieve high levels of transfection by incorporating a small amount of co-complexing agent. This approach could be especially beneficial for Poly-2 vectors, since cytotoxicity appears to be an important limitation for these vectors. To test this hypothesis, PLL and PEI were used as model co-complexing agents. We focused our attention on the most promising member in each of the Poly-1 (amine-terminated, $M_w$=13,100) and Poly-2 (amine-terminated, $M_w$=13,400) sets of polymers. The data displayed in FIGS. **23** and **24** indicate that a significant reduction in polymer could be achieved, while maintaining high levels of transfection efficiency, through the use of PLL or PEI as co-complexing agents. In some cases, significant enhancement of transfection activity was realized. As expected, this co-complexation approach was particularly beneficial for Poly-2 vectors. This work, and prior work (Wagner, E., et al., Influenza virus hemagglutinin HA-2 N-terminal fusogenic peptides augment gene transfer by transferrin-polylysine-DNA complexes: toward a synthetic virus-like gene-transfer vehicle. Proc. Natl. Acad. Sci. U.S.A., 1992. 89(17): p. 7934-8; Fritz, J. D., et al., Gene transfer into mammalian cells using histone-condensed plasmid DNA. Hum Gene Ther, 1996. 7(12): p. 1395-404; Pack, D. W., D. Putnam, and R. Langer, Design of imidazole-containing endosomolytic biopolymers for gene delivery. Biotechnol. Bioeng., 2000. 67(2): p. 217-23; Lim, Y. B., et al., Self-Assembled Ternary Complex of Cationic Dendrimer, Cucurbituril, and DNA: Noncovalent Strategy in Developing a Gene Delivery Carrier. Bioconjug Chem, 2002. 13(6): p. 1181-5; each of which is incorporated herein by reference), demonstrates that the blending of polymers with complementary gene transfer characteristics can in some cases produce more effective gene delivery reagents.

GFP Transfections

To further evaluate the transfection properties of the Poly-1/PLL and Poly-2/PLL blended reagents, we performed transfection experiments using a reporter plasmid coding for green fluorescent protein (pCMV-EGFP). Though the luciferase and GFP transfection assay systems both measure transfection activity, they provide different types of information. The luciferase system quantifies the total amount of exogenous luciferase protein produced by all the cells in a given well, providing a measure of cumulative transfection activity. In contrast, the GFP system can be used to quantify the percentage of cells that have been transfected, providing a cell-by-cell measure of transfection activity. Both systems are useful and offer complementary information regarding the transfection properties of a given gene delivery system.

GFP transfections were performed in a similar manner as the luciferase experiments, but were scaled up to 6-well plate format (5 μg plasmid/well). COS-7 cells were transfected using Poly-1/PLL (Poly-1:PLL:DNA=60:0.1:1 w/w/w) and Poly-2/PLL (Poly-2:PLL:DNA=15:0.4:1 w/w/w). Lipofectamine 2000 (μL reagent: μg DNA=1:1), PEI (PEI: DNA=1:1 w/w, N/P~8), and naked DNA were used as controls in the experiment. After 1 hr incubation with cells, vectors were removed and fresh growth media was added. Two days later GFP expression was assayed using a flow cytometer. Nearly all cells transfected with Poly-1/PLL were positive for GFP expression (FIGS. **25** and **26**). Experiments indicated that Poly-2/PLL vectors were less effective, resulting in roughly 25% positive cells. Positive controls Lipofectamine 2000 and PEI were also able to mediate effective transfection of COS-7 cells under the conditions employed. Although Lipofectamine 2000 and PEI transfections resulted in nearly the same percentage of GFP-positive cells as Poly-1/PLL, the fluorescence level of GFP-positive cells was higher for Poly-1/PLL (mean fluorescence=6033) than that of both Lipofectamine 2000 (mean fluorescence=5453) and PEI (mean fluorescence=2882). Multiplying the percentage of positive cells by the mean fluorescence level of positive cells provides a measure of aggregate expression for the sample and should, in theory, better correlate with the results of luciferase gene expression experiments. Quantifying total GFP expression in this manner indicates that the highest expression level is achieved by Poly-1/PLL, followed by Lipofectamine 2000 and PEI. This result is in general agreement with the luciferase expression results.

Experiments have shown that Poly-I/PLL (Poly-1:PLL: DNA=60:0.1:1 w/w/w) is a highly effective vector for transfecting COS-7 cells. The ability of this vector to mediate transfection in three other commonly used cell lines (CHO, NIH 3T3, and HepG2) was also investigated. It is very likely that each of these cell lines have optimal transfection conditions that differ from those used to transfect COS-7 cells; however, as a preliminary evaluation of the ability to transfect multiple cell lines, transfections were performed in the same manner and under the same conditions as the COS-7 transfections. Results indicate that Poly-1/PLL (Poly1:PLL: DNA=60:0.1:1 w/w/w) is able to successfully transfect CHO, NIH 3T3, and HepG2 cells, though not as effectively as COS-7 cells (FIG. **27**). This is not too surprising since the vector used was optimized by screening for gene transfer in COS-7 cells. Optimization of vector composition and transfection conditions specific for each cell type would be expected to result in even higher transfection levels.

SUMMARY

In this work, the role of polymer molecular weight, polymer chain end-group, and polymer/DNA ratio on a number of important gene transfer properties has been investigated. All three factors were found to have a significant impact on gene transfer, highlighting the benefit of carefully controlling and optimizing these parameters. In addition, the incorporation of a small amount of PLL, used to aid complexation, further enhances gene transfer. Through these approaches degradable poly(beta-amino esters)-based vectors that rival some of the best available non-viral vectors for in vitro gene transfer.

## Example 6

### Further Studies of Selected poly(beta-amino esters)

To further characterize and synthesize some of the poly (beta-amino esters) identified in previous screens, a twenty-one polymers were re-synthesized at various ratios of amine monomer to acrylate monomer. The resulting polymers were characterized by gel permeation chromatography to determine the molecular weight and polydispersitie of each polymer. Each of the polymer was then tested for its ability to transfect cells.

Polymer Synthesis. The polymers were synthesized as described in Example 5. Several versions of each polymer were created by varying the amin/diacrylated stoichiometric ratio. For example, C36-1 corresponds to the stoichiometric ratio of 1.4, and C36-12 to 0.6, with all the intermediates given in the table below:

| Version of C36 | Amine:Acrylate Stoichiometric Ratio |
|---|---|
| C36-1 | 1.4 |
| C36-2 | 1.3 |
| C36-3 | 1.2 |
| C36-4 | 1.1 |
| C36-5 | 1.05 |
| C36-6 | 1.025 |
| C36-7 | 1.0 |
| C36-8 | 0.975 |
| C36-9 | 0.950 |
| C36-10 | 0.9 |
| C36-11 | 0.8 |
| C36-12 | 0.6 |

The polymers were typically prepared in glass vials with no solvent at 100° C. for 5 hours. In some syntheses, the polymerization at 100° C. yielded highly cross-linked polymers when certain monomers such as amine 94 were used; therefore, the polymerization reactions were repeated at 50° C. with 2 mL of DMSO added to avoid cross-linking.

The resulting polymers were analyzed by GPC as described in Example 5. The molecular weights and polydispersities of each of the polymers is shown in the table below:



| Polymer | $M_w$ | $M_n$ | Polydispersity |
|---|---|---|---|
| F28-1 | 5540 | 2210 | 2.50678733 |
| F28-2 | 6150 | 2460 | 2.5 |
| F28-3 | 8310 | 2920 | 2.845890411 |
| F28-4 | 11600 | 3660 | 3.169398907 |
| F28-5 | 16800 | 4360 | 3.853211009 |
| F28-6 | 16100 | 4850 | 3.319587629 |
| F28-7 | 18000 | 5040 | 3.571428571 |
| F28-8 | 18200 | 5710 | 3.187390543 |
| F28-9 | 22300 | 7880 | 2.829949239 |
| F28-10 | 23700 | 8780 | 2.699316629 |
| F28-11 | 12100 | 5660 | 2.137809187 |
| F28-12 | 4850 | 2920 | 1.660958904 |
| C36-1 | 7080 | 3270 | 2.165137615 |
| C36-2 | 5100 | 2640 | 1.931818182 |
| C36-3 | 21200 | 8090 | 2.620519159 |
| C36-4 | 20500 | 6710 | 3.05514158 |
| C36-5 | 112200 | 33200 | 3.379518072 |
| C36-6 | 21700 | 6890 | 3.149492017 |
| C36-7 | 36800 | 15700 | 2.343949045 |
| C36-8 | 35700 | 12600 | 2.833333333 |
| C36-9 | 35200 | 15100 | 2.331125828 |
| C36-10 | 22500 | 9890 | 2.275025278 |
| C36-11 | 26000 | 6060 | 4.290429043 |
| D60-1 | 1890 | 1400 | 1.35 |
| D60-2 | 2050 | 1520 | 1.348684211 |
| D60-3 | 2670 | 1720 | 1.552325581 |
| D60-4 | 3930 | 2210 | 1.778280543 |
| D60-5 | 5130 | 2710 | 1.89298893 |
| D60-6 | 5260 | 2800 | 1.878571429 |
| D60-7 | 1130 | 1090 | 1.036697248 |
| D60-8 | 1840 | 1510 | 1.218543046 |
| D60-9 | 6680 | 3440 | 1.941860465 |
| D60-10 | 8710 | 4410 | 1.975056689 |
| D60-11 | 9680 | 4410 | 2.195011338 |
| D60-12 | 7450 | 3470 | 2.146974063 |
| D61-1 | 1710 | 1410 | 1.212765957 |
| D61-2 | 2600 | 1790 | 1.452513966 |
| D61-3 | 3680 | 2280 | 1.614035088 |
| D61-4 | 4630 | 2550 | 1.815686275 |
| D61-5 | NA | NA | NA |
| D61-6 | NA | NA | NA |
| D61-7 | 6110 | 3250 | 1.88 |
| D61-8 | 6410 | 3190 | 2.009404389 |
| D61-9 | 6790 | 3440 | 1.973837209 |
| D61-10 | 8900 | 4350 | 2.045977011 |
| D61-11 | 10700 | 4600 | 2.326086957 |
| D61-12 | 6760 | 2900 | 2.331034483 |
| F32-1 | 10300 | 3260 | 3.159509202 |
| F32-2 | 11100 | 3490 | 3.180515759 |
| F32-3 | 16600 | 4820 | 3.443983402 |
| F32-4 | 17300 | 5390 | 3.209647495 |
| F32-5 | 18600 | 5830 | 3.190394511 |
| F32-6 | 26200 | 8290 | 3.160434258 |
| C32-1 | 6670 | 2810 | 2.37366548 |
| C32-2 | 18100 | 5680 | 3.186619718 |
| C32-3 | 19300 | 6060 | 3.184818482 |
| C32-4 | 25600 | 9100 | 2.813186813 |
| C32-5 | 25000 | 7860 | 3.180661578 |
| C32-6 | 25700 | 8440 | 3.045023697 |
| C86-1 | 14200 | 2900 | 4.896551724 |
| C86-2 | 21000 | 2900 | 7.24137931 |
| C86-3 | 27500 | 4590 | 5.991285403 |
| U94-1 | 10700 | 3530 | 3.031161473 |
| U94-2 | NA | NA | NA |
| U94-3 | NA | NA | NA |
| F32-1 | 10300 | 3260 | 3.159509202 |
| F32-2 | 11100 | 3490 | 3.180515759 |
| F32-3 | 16600 | 4820 | 3.443983402 |
| F32-4 | 17300 | 5390 | 3.209647495 |
| F32-5 | 25000 | 7860 | 3.180661578 |
| F32-6 | 26200 | 8290 | 3.160434258 |
| C32-1 | 6670 | 2810 | 2.37366548 |
| C32-2 | 18100 | 5680 | 3.186619718 |
| C32-3 | 19300 | 6060 | 3.184818482 |
| C32-4 | 25600 | 9100 | 2.813186813 |
| C32-5 | 25000 | 7860 | 3.180661578 |
| C32-6 | 25700 | 8440 | 3.045023697 |
| U86-1 | Unusual | NA | NA |
| U86-2 | Unusual | NA | NA |
| U86-3 | Unusual | NA | NA |
| U86-4 | Unusual | NA | NA |
| U85-5 | Unusual | NA | NA |
| JJ32-1 | 9730 | 4010 | 2.426433915 |
| JJ32-2 | 12100 | 4580 | 2.641921397 |
| JJ32-3 | 19400 | 6510 | 2.980030722 |
| JJ32-4 | 27900 | 10000 | 2.79 |
| JJ32-5 | 32600 | 9720 | 3.353909465 |
| JJ32-6 | 28900 | 9870 | 2.928064843 |
| JJ36-1 | 7540 | 3550 | 2.123943662 |
| JJ36-2 | 143500 | 59600 | 2.407718121 |
| JJ36-3 | 20100 | 7310 | 2.749658003 |
| JJ36-4 | 30200 | 10200 | 2.960784314 |
| JJ36-5 | 33900 | 10600 | 3.198113208 |
| JJ36-6 | 36100 | 12500 | 2.888 |
| JJ28-1 | 7550 | 3240 | 2.330246914 |
| JJ28-2 | 9490 | 3460 | 2.742774566 |
| JJ28-3 | 16800 | 5420 | 3.099630996 |

-continued

| Polymer | $M_w$ | $M_n$ | Polydispersity |
|---|---|---|---|
| JJ28-4 | 23300 | 8090 | 2.880098888 |
| JJ28-5 | 25500 | 7700 | 3.311688312 |
| JJ28-6 | 32100 | 10900 | 2.944954128 |
| U28-1 | 7190 | 2580 | 2.786821705 |
| U28-2 | 10700 | 3990 | 2.681704261 |
| U28-3 | 15600 | 7300 | 2.136986301 |
| U28-4 | 20400 | 9880 | 2.064777328 |
| U28-5 | 20500 | 9670 | 2.119958635 |
| U28-6 | 24200 | 13000 | 1.861538462 |
| E28-1 | 5900 | 3280 | 1.798780488 |
| E28-2 | 7950 | 3550 | 2.23943662 |
| E28-3 | 14300 | 6300 | 2.26984127 |
| E28-4 | 6990 | 3320 | 2.105421687 |
| E28-5 | 17400 | 8180 | 2.127139364 |
| E28-6 | 19300 | 9030 | 2.137320044 |
| LL6-1 | 2380 | 1570 | 1.515923567 |
| LL6-2 | 3350 | 2070 | 1.618357488 |
| LL6-3 | 4110 | 2340 | 1.756410256 |
| LL6-4 | 5750 | 3010 | 1.910299003 |
| LL6-5 | 7810 | 5050 | 1.546534653 |
| LL6-6 | 6950 | 4190 | 1.658711217 |
| LL8-1 | 3160 | 1910 | 1.654450262 |
| LL8-2 | 3630 | 2560 | 1.41796875 |
| LL8-3 | 5300 | 3520 | 1.505681818 |
| LL8-4 | 6000 | 3320 | 1.807228916 |
| LL8-5 | 8160 | 4730 | 1.725158562 |
| LL8-6 | 7190 | 4650 | 1.546236559 |
| U36-1 | 7290 | 3370 | 2.163204748 |
| U36-2 | 11100 | 5000 | 2.22 |
| U36-3 | 12600 | 5470 | 2.303473492 |
| U36-4 | 21500 | 8550 | 2.514619883 |
| U36-5 | 24700 | 9430 | 2.619300106 |
| U36-6 | 31700 | 10700 | 2.962616822 |
| E36-1 | 6030 | 3130 | 1.926517572 |
| E36-2 | 8510 | 4040 | 2.106435644 |
| E36-3 | 12800 | 5730 | 2.233856894 |
| E36-4 | 18200 | 7620 | 2.388451444 |
| E36-5 | 20100 | 8050 | 2.49689441 |
| E36-6 | 32900 | 10900 | 3.018348624 |
| U32-1 | 9830 | 3790 | 2.593667546 |
| U32-2 | 12000 | 4460 | 2.69058296 |
| U32-3 | 18200 | 6780 | 2.684365782 |
| U32-4 | 25200 | 11100 | 2.27027027 |
| U32-5 | 26500 | 9360 | 2.831196581 |
| U32-6 | 26200 | 10600 | 2.471698113 |
| E32-1 | 7070 | 3310 | 2.135951662 |
| E32-2 | 9920 | 4180 | 2.373205742 |
| E32-3 | 14700 | 6080 | 2.417763158 |
| E32-4 | 23500 | 9160 | 2.565502183 |
| E32-5 | 28800 | 10000 | 2.88 |
| E32-6 | 26900 | 10300 | 2.611650485 |
| C94-1 | 6760 | 3110 | 2.173633441 |
| C94-2 | 10800 | 4190 | 2.577565632 |
| C94-3 | 18000 | 5330 | 3.377110694 |
| C94-4 | 38900 | 6660 | 5.840840841 |
| C94-5 | Didn't dissolve | NA | NA |
| C94-6 | Didn't dissolve | NA | NA |
| D94-1 | 6030 | 2980 | 2.023489933 |
| D94-2 | 6620 | 3370 | 1.964391691 |
| D94-3 | 9680 | 3950 | 2.450632911 |
| D94-4 | 11500 | 4510 | 2.549889135 |
| D94-5 | 13700 | 4940 | 2.773279352 |
| D94-6 | 18800 | 5650 | 3.327433628 |
| F94-1 | 5570 | 2740 | 2.032846715 |
| F94-2 | 7670 | 3180 | 2.411949686 |
| F94-3 | 12600 | 4230 | 2.978723404 |
| F94-4 | 20300 | 5160 | 3.934108527 |
| F94-5 | 21500 | 5390 | 3.988868275 |
| F94-6 | 27300 | 6310 | 4.326465927 |
| JJ94-1 | 7750 | 3360 | 2.306547619 |
| JJ94-2 | 12700 | 4590 | 2.766884532 |
| JJ94-3 | 30500 | 7280 | 4.18956044 |
| JJ94-4 | Didn't dissolve | NA | NA |
| JJ94-5 | Didn't dissolve | NA | NA |
| JJ94-6 | Didn't dissolve | NA | NA |
| F86-1 | 3940 | 2630 | 1.498098859 |
| F86-2 | 5300 | 3190 | 1.661442006 |
| F86-3 | 7790 | 4040 | 1.928217822 |



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| Polymer | $M_w$ | $M_n$ | Polydispersity |
|---|---|---|---|
| F86-4 | 11000 | 5410 | 2.033271719 |
| F86-5 | 10600 | 5650 | 1.876106195 |
| F86-6 | 13300 | 6440 | 2.065217391 |
| D86-1 | 4610 | 2830 | 1.628975265 |
| D86-2 | 5570 | 3290 | 1.693009119 |
| D86-3 | 7120 | 3770 | 1.888594164 |
| D86-4 | 8310 | 4440 | 1.871621622 |
| D86-5 | 8950 | 4710 | 1.900212314 |
| D86-6 | 10400 | 5010 | 2.075848303 |
| U86-1 | 5940 | 3500 | 1.697142857 |
| U86-2 | 7780 | 4430 | 1.756207675 |
| U86-3 | 11900 | 6540 | 1.819571865 |
| U86-4 | 15100 | 7630 | 1.979030144 |
| U86-5 | 16300 | 8950 | 1.82122905 |
| U86-6 | 18100 | 9810 | 1.845056065 |
| E86-1 | 4880 | 3140 | 1.554140127 |
| E86-2 | 6300 | 3790 | 1.662269129 |
| E86-3 | 9780 | 5140 | 1.902723735 |
| E86-4 | 12500 | 6350 | 1.968503937 |
| E86-5 | 13400 | 6820 | 1.964809384 |
| E86-6 | 15500 | 7280 | 2.129120879 |
| JJ86-1 | 5460 | 3370 | 1.620178042 |
| JJ86-2 | 6880 | 4080 | 1.68627451 |
| JJ86-3 | 11900 | 6180 | 1.925566343 |
| JJ86-4 | 14200 | 7000 | 2.028571429 |
| JJ86-5 | 20500 | 9090 | 2.255225523 |
| JJ86-6 | 16300 | 7770 | 2.097812098 |
| C86-1 | 4870 | 3030 | 1.607260726 |
| C86-2 | 5720 | 3460 | 1.653179191 |
| C86-3 | 9970 | 5060 | 1.970355731 |
| C86-4 | 14200 | 7000 | 2.028571429 |
| C86-5 | 17700 | 8500 | 2.082352941 |
| C80-6 | 12600 | 6050 | 2.082644628 |
| E80-1 | 2840 | 2010 | 1.412935323 |
| E80-2 | 3720 | 2420 | 1.537190083 |
| E80-3 | 6080 | 3650 | 1.665753425 |
| E80-4 | 7210 | 4240 | 1.700471698 |
| E80-5 | 7640 | 4290 | 1.780885781 |
| E80-6 | 9000 | 5310 | 1.694915254 |
| JJ80-1 | 3410 | 2180 | 1.564220183 |
| JJ80-2 | 4590 | 2890 | 1.588235294 |
| JJ80-3 | 8430 | 4750 | 1.774736842 |
| JJ80-4 | 11300 | 6560 | 1.722560976 |
| JJ80-5 | 13200 | 7160 | 1.843575419 |
| JJ80-6 | 11600 | 6540 | 1.773700306 |
| U80-1 | 4300 | 2680 | 1.604477612 |
| U80-2 | 5130 | 3020 | 1.698675497 |
| U80-3 | 8320 | 4700 | 1.770212766 |
| U80-4 | 9130 | 4880 | 1.870901639 |
| U80-5 | 11300 | 5750 | 1.965217391 |
| U80-6 | 11200 | 5920 | 1.891891892 |

Luciferase Transfection Assay. As described in Example 5, COS-7 cells were transfected with pCMV-Luc DNA using each of the polymers at polymer-to-DNA ratios ranging from 10:1 to 100:1 (weight:weight). Luciferase expresseion was analysed using Bright-Glo assay kits (Promega). Luminescence was measured for each transfection, and the luminescence was used to calculate nanograms of Luciferase enzyme as described in Example 5. Experiments were done in quadruplicate, and the values shown in the tables below are the averaged values from the four experiments. These data are shown below for each polymer synthesized.

| C36-1 | C36-2 | C36-3 | C36-4 | C36-5 | C36-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.168527 | 0.345149 | 0.627992 | 0.152258 | 0.068355 | 0.094073 | 10 |
| 3.58467 | 0.12639 | 21.27867 | 2.145388 | 0.163042 | 0.184298 | 20 |
| 4.295966 | 0.927605 | 18.84046 | 4.750661 | 0.287989 | 1.063834 | 30 |
| 7.150343 | 1.137242 | 17.04771 | 7.529555 | 0.080757 | 0.332789 | 40 |
| 3.74705 | 1.180274 | 6.875879 | 9.710764 | 0.582186 | 1.963895 | 60 |
| 0.705683 | 0.212297 | 0.560245 | 7.221382 | 5.003849 | 5.813189 | 100 |
| **C36-7** | **C36-8** | **C36-9** | **C36-10** | **C36-11** | **C-36-12** | **Ratio** |
| 0.164373 | 0.085336 | 0.116502 | 0.042173 | 0.062905 | 0.18877 | 10 |
| 0.134132 | 0.096043 | 0.091152 | 0.032851 | 0.032115 | 0.383965 | 20 |
| 0.020768 | 0.021203 | 0.0665 | 0.021953 | 0.017807 | 0.288102 | 30 |
| 0.05027 | 0.060731 | 0.017768 | 0.011885 | 0.008923 | 0.128469 | 40 |
| 0.031233 | 0.048807 | 0.025626 | 0.012516 | 0.002606 | 0.213173 | 60 |
| 0.116587 | 0.129504 | 0.332497 | 0.123413 | 0.058442 | 0.250708 | 100 |

| C86-1 | C86-2 | C86-3 | Ratio |
|---|---|---|---|
| 0.157713 | 0.475708 | 1.093272 | 10 |
| 0.242481 | 0.616621 | 1.439904 | 20 |
| 0.396888 | 0.992601 | 1.758045 | 30 |
| 0.300173 | 1.276707 | 1.901677 | 40 |

| D60-1 | D60-2 | D60-3 | D60-4 | D60-5 | D60-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.604984 | 0.443875 | 0.363271 | 0.260475 | 0.498462 | 0.466087 | 10 |
| 0.115174 | 0.174976 | 0.250613 | 0.40783 | 0.587186 | 0.89381 | 20 |
| 0.138372 | 0.45915 | 0.81101 | 0.773161 | 1.264634 | 1.438474 | 30 |
| 0.135287 | 0.506303 | 2.344053 | 1.695591 | 2.302305 | 2.959638 | 40 |
| 0.203804 | 0.679718 | 3.908348 | 2.216808 | 3.129304 | 4.335511 | 60 |
| 0.233546 | 0.640246 | 0.251146 | 3.112999 | 7.65786 | 6.759895 | 100 |
| **D60-7** | **D60-8** | **D60-9** | **D60-10** | **D60-11** | **D60-12** | **Ratio** |
| 0.299777 | 0.333863 | 0.434027 | 0.46862 | 0.387458 | 0.211083 | 10 |
| 0.237477 | 0.266398 | 1.211246 | 1.385232 | 1.034892 | 0.215027 | 20 |
| 0.339709 | 0.665539 | 2.958346 | 5.607664 | 3.514454 | 0.485295 | 30 |
| 0.499842 | 1.216181 | 4.406196 | 6.736276 | 5.121445 | 0.444359 | 40 |
| 1.297394 | 1.009228 | 5.951785 | 9.565956 | 7.193687 | 0.35831 | 60 |
| 5.399266 | 0.135852 | 5.725666 | 10.45568 | 5.414051 | 0.245279 | 100 |

| D61-1 | D61-2 | D61-3 | D61-4 | D61-5 | D61-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.329886 | 0.29803 | 0.190101 | 0.142813 | 0.114565 | 0.227593 | 10 |
| 0.299409 | 0.710035 | 0.295508 | 0.288845 | 0.247909 | 0.32839 | 20 |
| 0.155568 | 0.680763 | 0.618022 | 0.651633 | 0.402721 | 1.831437 | 30 |
| 0.085824 | 0.620294 | 3.722971 | 4.572264 | 3.010274 | 11.69027 | 40 |
| 0.188357 | 0.187979 | 3.970054 | 7.147033 | 10.85674 | 9.238981 | 60 |
| 0.019321 | 0.001369 | 0.034958 | 0.033062 | 0.202601 | 0.131544 | 100 |
| **D61-7** | **D61-8** | **D61-9** | **D61-10** | **D61-11** | **D61-12** | **Ratio** |
| 0.153122 | 0.180646 | 0.1073 | 0.244713 | 0.231561 | 0.18571 | 10 |
| 0.203312 | 0.217288 | 0.191108 | 0.185759 | 0.270723 | 0.119897 | 20 |
| 0.539455 | 0.239807 | 0.140418 | 0.174014 | 0.320869 | 0.094186 | 30 |
| 1.679507 | 1.020126 | 0.584908 | 0.229946 | 0.474142 | 0.154025 | 40 |
| 2.69543 | 5.9829 | 7.008946 | 1.308281 | 0.301803 | 0.067526 | 60 |
| 1.271189 | 2.402989 | 3.186707 | 5.576734 | 1.343239 | 0.115366 | 100 |

| U94-1 | U94-2 | U94-3 | Ratio |
|---|---|---|---|
| 0.233894 | 0.127165 | 0.804911 | 10 |
| 0.179855 | 1.35532 | 13.53974 | 20 |
| 0.275078 | 16.26098 | 20.65427 | 30 |

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| U94-1 | U94-2 | U94-3 | Ratio |
|---|---|---|---|
| 1.161574 | 19.93922 | 13.08098 | 40 |
| 1.961067 | 18.39299 | 9.319949 | 60 |
| 13.0485 | 7.591092 | 1.647718 | 100 |

| C32-1 | C32-2 | C32-3 | C32-4 | C32-5 | C32-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.137436 | 0.544141 | 0.138034 | 0.112832 | 0.087552 | 0.131699 | 10 |
| 0.159782 | 28.93062 | 14.3276 | 0.316178 | 0.125792 | 0.242881 | 20 |
| 0.166661 | 53.90695 | 24.83791 | 0.67551 | 0.193545 | 0.181321 | 30 |
| 0.392402 | 90.62006 | 49.11244 | 2.271509 | 0.563168 | 0.632798 | 40 |
| 6.034825 | 73.59378 | 46.31 | 2.490156 | 0.111248 | 0.273411 | 60 |
| 38.17463 | 60.21433 | 51.86994 | 16.43407 | 2.01284 | 2.619288 | 100 |

| F32-1 | F32-2 | F32-3 | F32-4 | F32-5 | F32-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.746563 | 2.446604 | 1.288067 | 0.210478 | 0.202798 | 0.112283 | 10 |
| 20.84138 | 20.94165 | 11.46963 | 1.780569 | 10.90572 | 0.100889 | 20 |
| 23.8042 | 23.7095 | 13.34488 | 5.01115 | 9.510119 | 0.255589 | 30 |
| 17.47681 | 17.35353 | 14.74619 | 8.361793 | 6.436393 | 0.599084 | 40 |
| 10.54807 | 11.78762 | 13.58168 | 7.499322 | 4.865577 | 0.322946 | 60 |
| 0.072034 | 0.090408 | 0.332458 | 2.300951 | 2.434663 | 1.644695 | 100 |

| F28-1 | F28-2 | F28-3 | F28-4 | F28-5 | F28-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.245612 | 0.247492 | 0.140455 | 0.203674 | 0.09426 | 0.131075 | 10 |
| 0.464885 | 0.192584 | 0.217777 | 0.213391 | 0.171565 | 0.397716 | 20 |
| 0.290643 | 0.19239 | 0.396845 | 0.433955 | 0.361789 | 2.02073 | 30 |
| 0.325066 | 0.189405 | 1.048323 | 2.088649 | 3.888705 | 19.95507 | 40 |
| 0.108766 | 0.164709 | 13.95859 | 0.411927 | 7.851029 | 21.77709 | 60 |
| 0.163978 | 6.619239 | 6.832291 | 8.409421 | 6.682506 | 2.958283 | 100 |
| **F28-7** | **F28-8** | **F28-9** | **F28-10** | **F28-11** | **F28-12** | **Ratio** |
| 0.094505 | 0.043474 | 0.05224 | 0.050384 | 0.104016 | 0.149513 | 10 |
| 0.173987 | 0.098512 | 0.069287 | 0.057704 | 0.131067 | 0.028219 | 20 |
| 0.705917 | 0.254424 | 0.083005 | 0.04454 | 0.133842 | 0.001619 | 30 |
| 2.860034 | 0.928959 | 0.226468 | 0.076503 | 0.095093 | 0.003896 | 40 |
| 7.786755 | 1.932506 | 0.42898 | 0.028744 | 0.063298 | 0.001342 | 60 |
| 8.655579 | 12.729 | 1.396803 | 0.281853 | 0.115513 | 0.001145 | 100 |

| JJ28-1 | JJ28-2 | JJ28-3 | JJ28-4 | JJ28-5 | JJ28-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.122351 | 0.056864 | 0.030798 | 0.041065 | 0.02373 | 0.051849 | 10 |
| 0.060598 | 0.059073 | 22.46575 | 2.229717 | 0.076134 | 0.053754 | 20 |
| 0.174243 | 0.211589 | 21.92396 | 4.089533 | 0.121043 | 0.115906 | 30 |
| 0.133603 | 36.42899 | 69.60415 | 19.16868 | 0.292215 | 0.337099 | 40 |
| 1.011778 | 64.69601 | 71.50927 | 47.49171 | 0.755335 | 0.654333 | 60 |
| 60.46546 | 56.7025 | 53.44758 | 39.13032 | 25.81403 | 3.936471 | 100 |

| JJ36-1 | JJ36-2 | JJ36-3 | JJ36-4 | JJ36-5 | JJ36-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.506359 | 1.634649 | 0.146132 | 0.053268 | 0.035023 | 0.033605 | 10 |
| 2.185596 | 4.332834 | 0.677853 | 0.017846 | 0.010687 | 0.004264 | 20 |
| 2.339652 | 5.039758 | 0.773873 | 0.024164 | 0.06932 | 0.009318 | 30 |
| 0.681878 | 1.871844 | 1.539743 | 0.087428 | 0.017886 | 0.009752 | 40 |
| 0.521703 | 1.592328 | 2.000554 | 0.201203 | 0.027165 | 0.004975 | 60 |
| 0.003277 | 0.067895 | 1.031285 | 0.284902 | 0.159879 | 0.008844 | 100 |

| JJ32-1 | JJ32-2 | JJ32-3 | JJ32-4 | JJ32-5 | J332-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.392821 | 1.158486 | 0.191533 | 0.127891 | 0.099083 | 0.076569 | 10 |
| 17.51289 | 21.24103 | 1.803172 | 0.065286 | 0.160362 | 0.108887 | 20 |
| 38.08705 | 43.77517 | 24.76927 | 0.09612 | 0.063692 | 0.044659 | 30 |
| 25.9567 | 34.88211 | 26.36994 | 0.201907 | 0.015214 | 0.026165 | 40 |
| 11.37519 | 17.48944 | 29.59326 | 0.175101 | 0.052321 | 0.098545 | 60 |
| 1.3311 | 1.288845 | 6.86144 | 0.70071 | 0.178921 | 0.70361 | 100 |

| LL6-1 | LL6-2 | LL6-3 | LL6-4 | LL6-5 | LL6-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.405305 | 0.583416 | 0.520853 | 0.50183 | 0.656802 | 1.060478 | 10 |
| 0.411758 | 0.747731 | 0.460075 | 0.287671 | 0.382454 | 1.099616 | 20 |
| 0.809416 | 0.377302 | 1.253481 | 1.099976 | 2.59164 | 1.138122 | 30 |
| 0.475903 | 0.854576 | 1.812577 | 2.018906 | 2.837056 | 0.69298 | 40 |
| 0.139647 | 0.29034 | 0.939013 | 1.992525 | 2.250511 | 3.059824 | 60 |
| 0.00154 | 0.002408 | 0.223682 | 2.932931 | 3.939451 | 6.879564 | 100 |

| LL8-1 | LL8-2 | LXS-3 | LL8-4 | LL8-5 | LX8-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.533009 | 1.180815 | 1.581011 | 2.254195 | 1.73015 | 1.76882 | 10 |
| 1.174539 | 1.228513 | 1.002632 | 2.369943 | 1.958308 | 2.928439 | 20 |
| 1.182611 | 1.620962 | 3.771897 | 3.988759 | 3.936124 | 0.000474 | 30 |
| 1.366191 | 1.875091 | 4.594308 | 4.253834 | 4.07168 | 0.000948 | 40 |
| 0.120086 | 0.866135 | 1.925861 | 4.423822 | 4.081074 | 5.083137 | 60 |
| 0.003316 | 0.029499 | 0.336777 | 4.077347 | 4.416413 | 4.241522 | 100 |

| U28-1 | U28-2 | U28-3 | U28-4 | U28-5 | U28-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.049477 | 0.044465 | 0.045254 | 0.034669 | 0.031628 | 0.025942 | 10 |
| 0.111915 | 0.050661 | 0.03988 | 0.015399 | 0.049004 | 0.020729 | 20 |
| 0.041895 | 0.050582 | 0.048212 | 0.0649171 | 0.069067 | 0.02756 | 30 |
| 0.122271 | 0.078429 | 1.647405 | 0.4885611 | 1.036216 | 0.058913 | 40 |
| 0.059982 | 0.051095 | 18.03734 | 5.718868 | 1.991446 | 0.176516 | 60 |
| 0.059585 | 44.91463 | 51.17075 | 34.01612 | 32.39362 | 3.488256 | 100 |

| E28-1 | E28-2 | E28-3 | E28-4 | E28-5 | E28-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.109892 | 0.150078 | 0.630192 | 0.187585 | 0.311968 | 0.168724 | 10 |
| 0.088189 | 0.509429 | 0.589377 | 0.106743 | 0.70723 | 0.144608 | 20 |
| 1.672278 | 12.4166 | 21.41889 | 3.405963 | 8.337341 | 1.32881 | 30 |
| 5.658186 | 12.17055 | 13.3351 | 7.272109 | 17.92266 | 5.089686 | 40 |
| 6.016333 | 5.424512 | 5.549474 | 5.185252 | 15.62457 | 8.706483 | 60 |
| 1.146098 | 0.804227 | 0.592348 | 0.529551 | 1.752402 | 5.438448 | 100 |

| U36-1 | U36-2 | U36-3 | U36-4 | U36-5 | U36-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.158334 | 0.388325 | 0.255439 | 0.139618 | 0.069735 | 0.054654 | 10 |
| 0.281155 | 2.119615 | 0.34803 | 0.196109 | 0.111805 | 0.067406 | 20 |
| 0.968904 | 2.501669 | 3.74982 | 0.370814 | 0.103744 | 0.077397 | 30 |
| 0.559332 | 1.595139 | 4.650123 | 0.65127 | 0.078343 | 0.029797 | 40 |
| 0.565322 | 0.540675 | 4.182981 | 1.59494 | 0.250723 | 0.029297 | 60 |
| 0.238507 | 0.008686 | 1.052448 | 2.269889 | 1.310025 | 0.23654 | 100 |

| E36-1 | E36-2 | E36-3 | E36-4 | E36-5 | E36-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.130066 | 2.940734 | 0.350723 | 0.077836 | 0.051293 | 0.018123 | 10 |
| 0.496911 | 1.866482 | 2.236257 | 0.135236 | 0.063655 | 0.020018 | 20 |
| 1.044698 | 0.617286 | 4.27308 | 0.882725 | 0.187495 | 0.01532 | 30 |
| 0.342085 | 0.025849 | 1.935655 | 1.170393 | 0.23494 | 0.004896 | 40 |
| 0.112427 | 0.211108 | 1.068847 | 1.362371 | 0.628612 | 0.070175 | 60 |
| 0.002566 | 0.003672 | 0.131476 | 1.367514 | 1.194649 | 0.11883 | 100 |

| U32-1 | U32-2 | U32-3 | U32-4 | U32-5 | U32-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.202681 | 0.107654 | 0.035536 | 0.037195 | 0.041027 | 0.047701 | 10 |
| 0.106511 | 0.084192 | 0.067327 | 0.012951 | 0.028982 | 0.012003 | 20 |
| 1.497135 | 2.867785 | 1.828273 | 0.056586 | 0.033682 | 0.010305 | 30 |
| 4.41159 | 13.8534 | 0.272404 | 0.056588 | 0.029377 | 0.021045 | 40 |
| 17.28347 | 38.37457 | 3.026925 | 0.017452 | 0.015556 | 0.060968 | 60 |
| 11.81885 | 13.6584 | 15.232970.673546 | | 0.121004 | 0.15261 | 100 |

| E32-1 | E32-2 | E32-3 | E32-4 | E32-5 | E32-6 | Ratio |
|---|---|---|---|---|---|---|
| 0.481414 | 0.898699 | 0.149685 | 0.093788 | 0.086886 | 0.046001 | 10 |
| 5.170892 | 6.057429 | 1.52106 | 0.054373 | 0.099557 | 0.01378 | 20 |
| 0.965091 | 2.279423 | 4.380769 | 0.112159 | 0.027166 | 0.038894 | 30 |
| 0.848062 | 1.086906 | 3.971834 | 0.13767 | 0.068236 | 0.090555 | 40 |
| 0.225141 | 0.688091 | 2.653561 | 0.570809 | 0.080796 | 0.01603 | 60 |
| 0.046762 | 0.176583 | 0.897883 | 1.365759 | 0.845009 | 0.111133 | 100 |

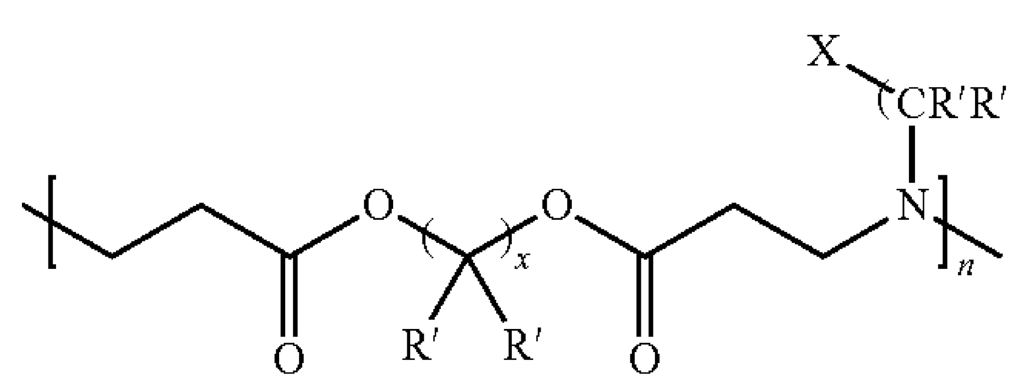
| C94-1 | C94-2 | C94-3 | C94-4 | Ratio |
|---|---|---|---|---|
| 0.289113 | 0.086166 | 0.151651 | 0.203119 | 10 |
| 0.133487 | 0.045908 | 0.067867 | 7.650297 | 20 |
| 0.293536 | 0.086328 | 0.853319 | 10.31612 | 30 |
| 0.198737 | 0.170611 | 1.955433 | 9.745005 | 40 |
| 0.312808 | 0.908991 | 3.536115 | 3.580573 | 60 |
| 0.32801 | 0.853063 | 2.414853 | 1.731594 | 100 |

## Other Embodiments

The foregoing has been a description of certain non-limiting preferred embodiments of the invention. Those of ordinary skill in the art will appreciate that various changes and modifications to this description may be made without departing from the spirit or scope of the present invention, as defined in the following claims.

What is claimed is:

**1**. A polymer of the formula:



wherein X is **[**methyl,**]** OR or **[**NR2**]** $NR_2$;

R is selected from the group consisting of hydrogen, **[**alkyl,**]** alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;

each R' is independently selected from the group consisting of hydrogen, **[**C1-C6**]** $C_1$-$C_6$ lower alkyl, **[**C1-C6**]** $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;

n is an integer between 3 and 10,000;

x is an integer between **[**1**]** *5* and 10;

y is an integer between 1 and 10; and salts thereof.

**2**. The polymer of claim **1**, wherein the polymer is amine-terminated.

**3**. The polymer of claim **1**, wherein the polymer is acrylate-terminated.

**4**. The polymer of claim **1**, wherein x is an integer between **[**2**]** *5* and 7.

**5**. The polymer of claim **1**, wherein x is **[**4**]** *5*.

**6**. The polymer of claim **1**, wherein x is **[**5**]** *6*.

**7**. The polymer of claim **1**, wherein x is **[**6**]** *7*.

**8**. The polymer of claim **1**, wherein y is an integer between 2 and 7.

**9**. The polymer of claim **1**, wherein y is 4.

**10**. The polymer of claim **1**, wherein y is 5.

**11**. The polymer of claim **1**, wherein y is 6.

**12**. The polymer of claim **1**, wherein R is hydrogen.

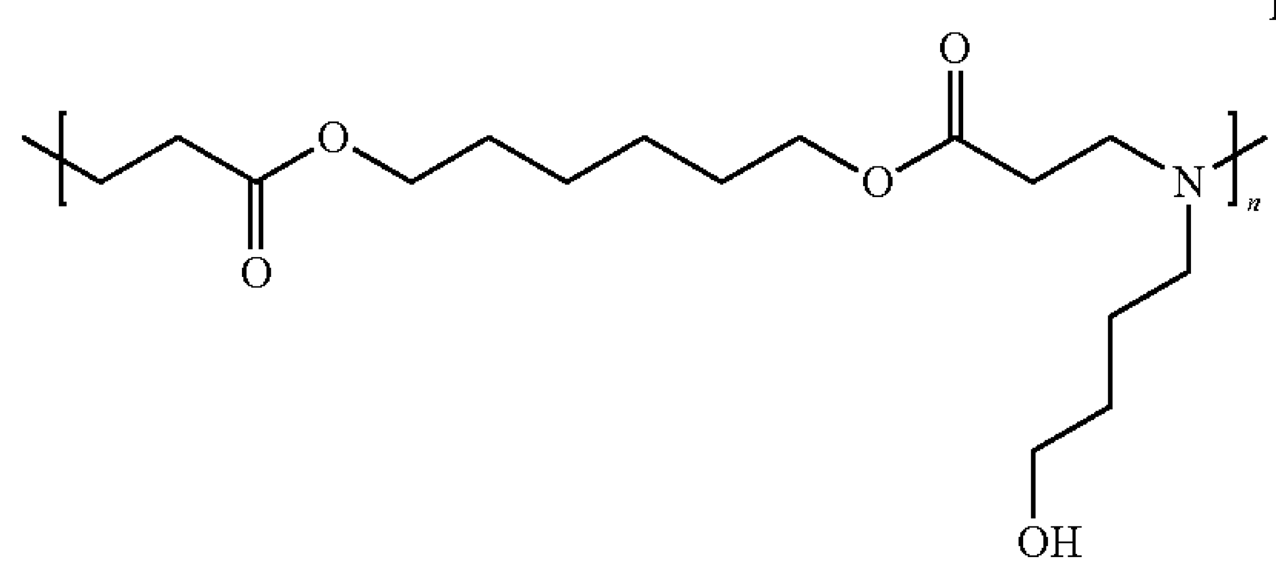
**[13**. The polymer of claim **1**, wherein R is methyl, ethyl, propyl, butyl, pentyl, and hexyl.**]**

**[14**. The polymer of claim **1**, wherein the structure of the compound is

wherein R is hydrogen, x is 4, and y is 4.**]**

**15**. The polymer of claim **1**, wherein the structure of the **[**compound**]** *polymer* is

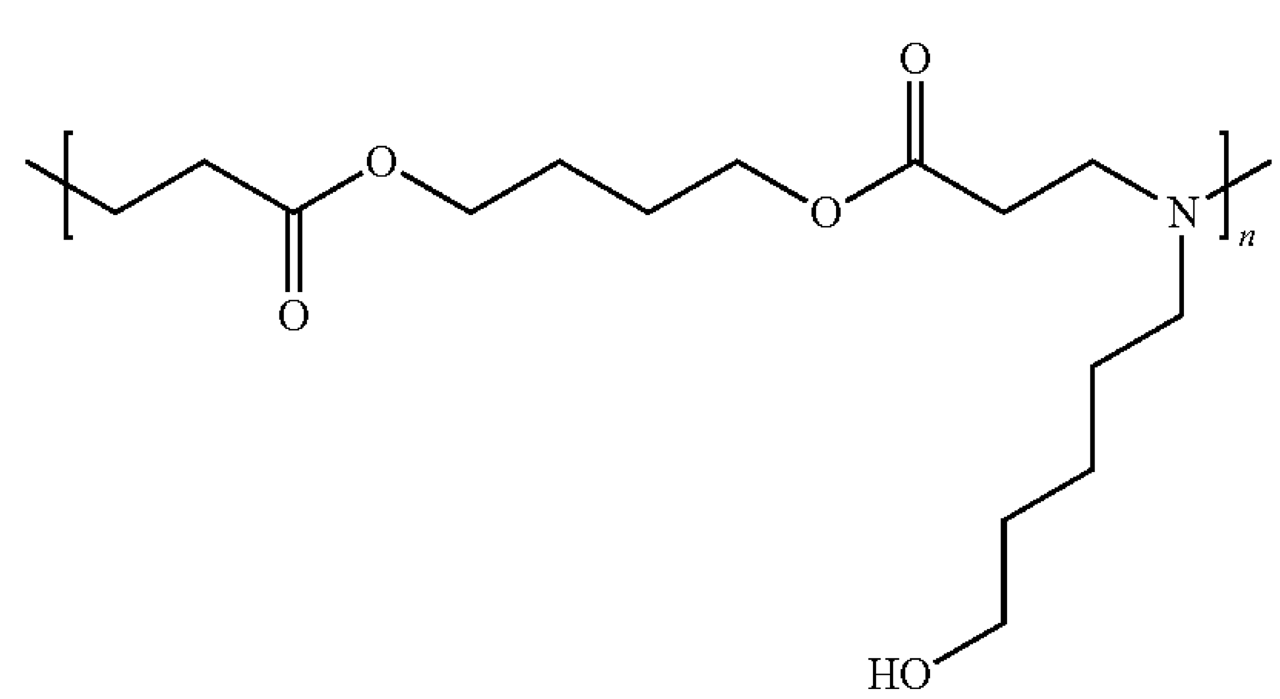
E28



wherein R is hydrogen, x is 6, and y is 4.

**16**. The polymer of claim **[1]** *37*, wherein the structure of the **[**compound**]** *polymer* is

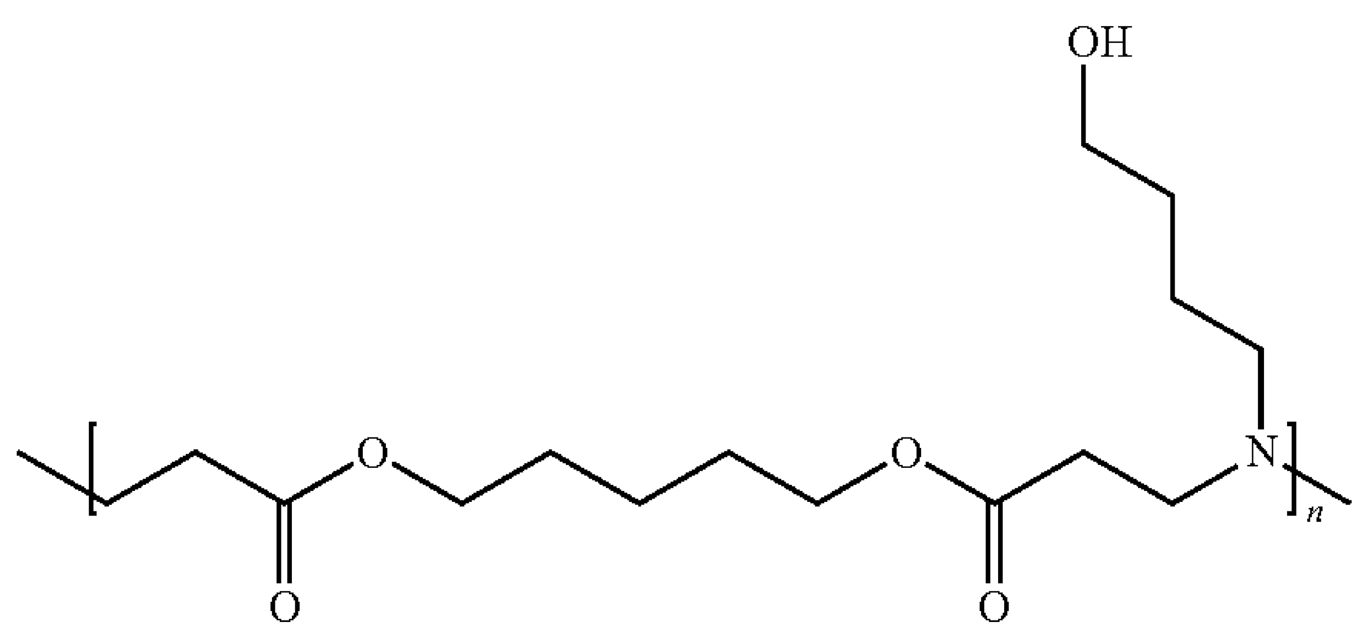
C32



wherein R is hydrogen, x is 4, and y is 5.

**17**. The polymer of claim **1**, wherein the structure of the **[**compound**]** *polymer* is

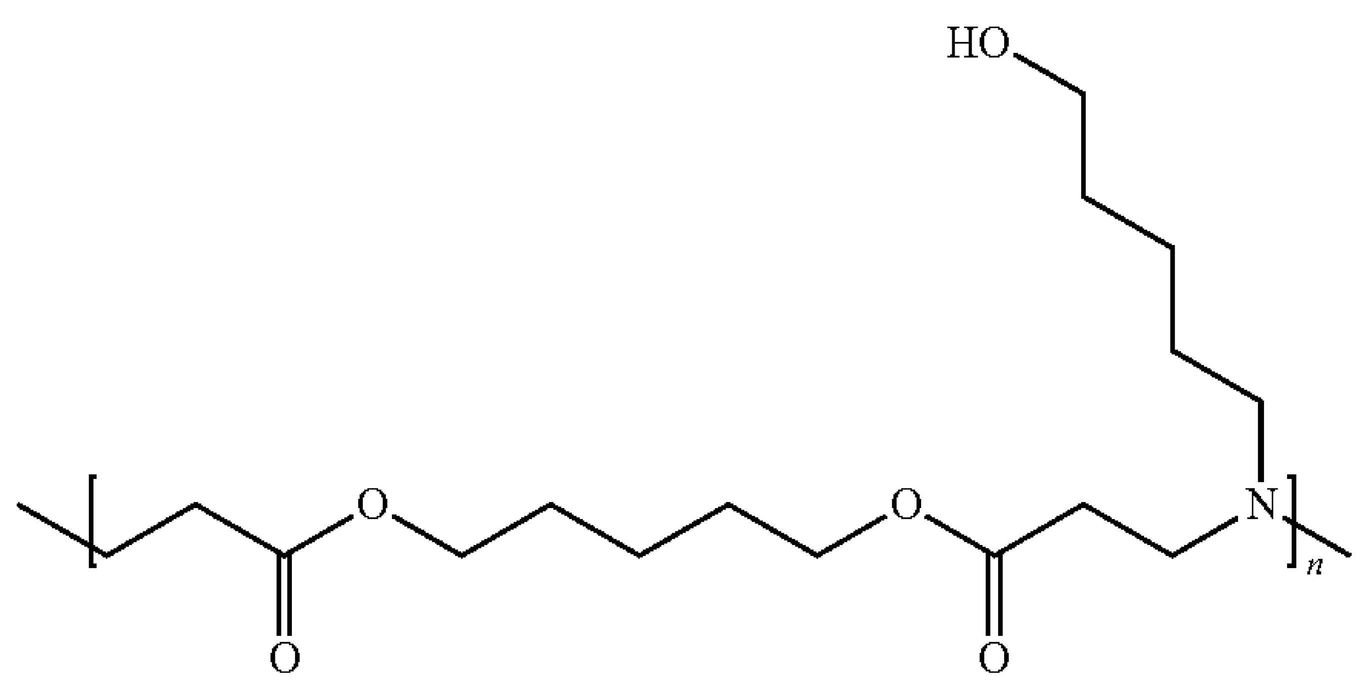
JJ28



wherein R is hydrogen, x is 5, and y is 4.

**18**. The polymer of claim **1**, wherein the structure of the **[**compound**]** *polymer* is

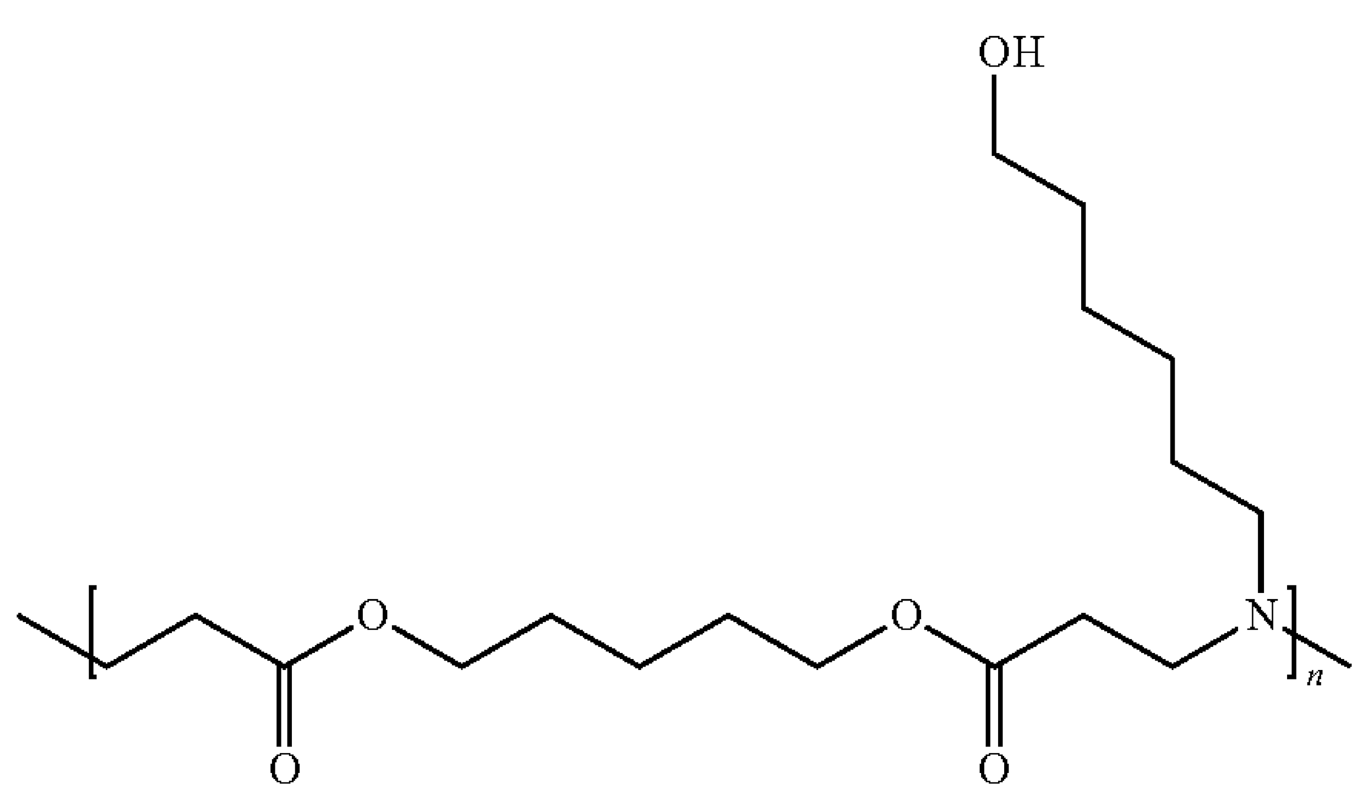
JJ32



wherein R is hydrogen, x is 5, and y is 5.

**19**. The polymer of claim **1**, wherein the structure of the **[**compound**]** *polymer* is

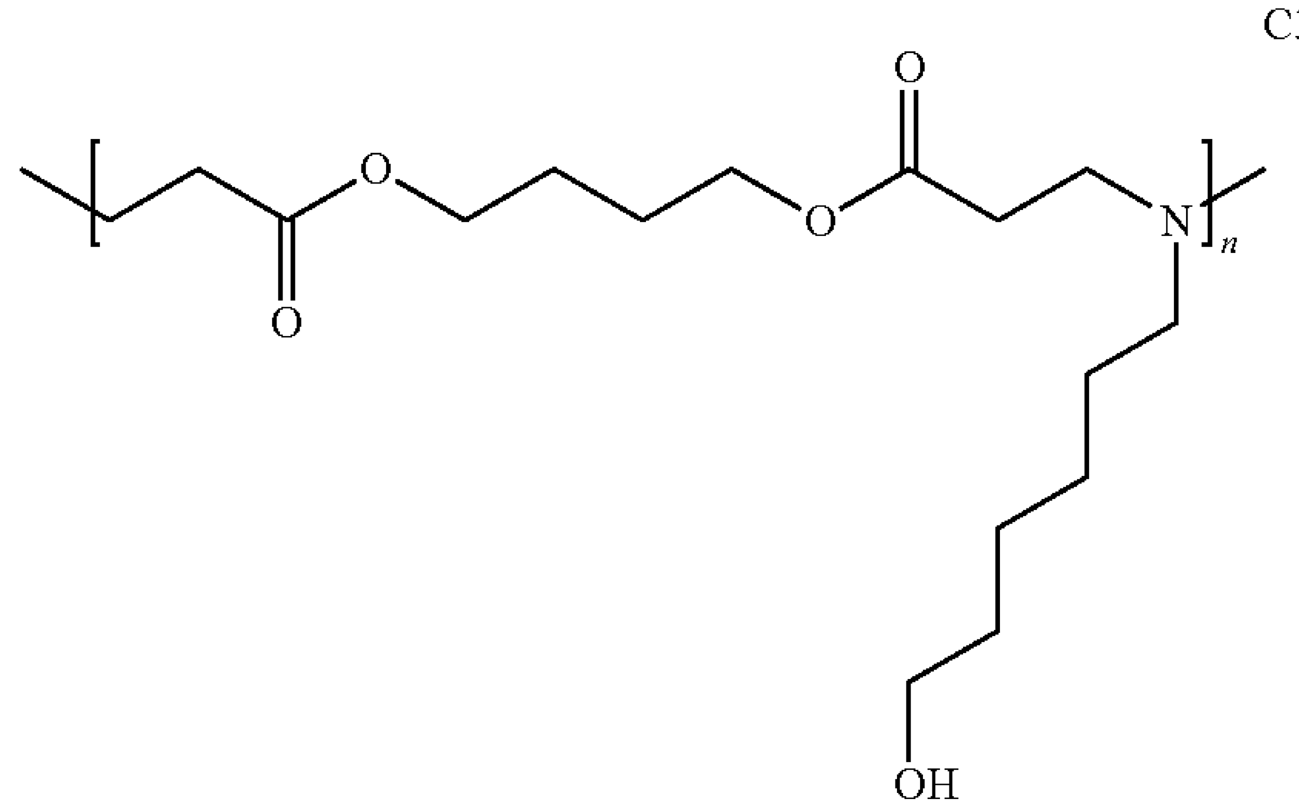
JJ36



wherein R is hydrogen, x is 5, and y is 6.

**20**. **[**A**]** *The* polymer of *claim 37* the formula:

C36



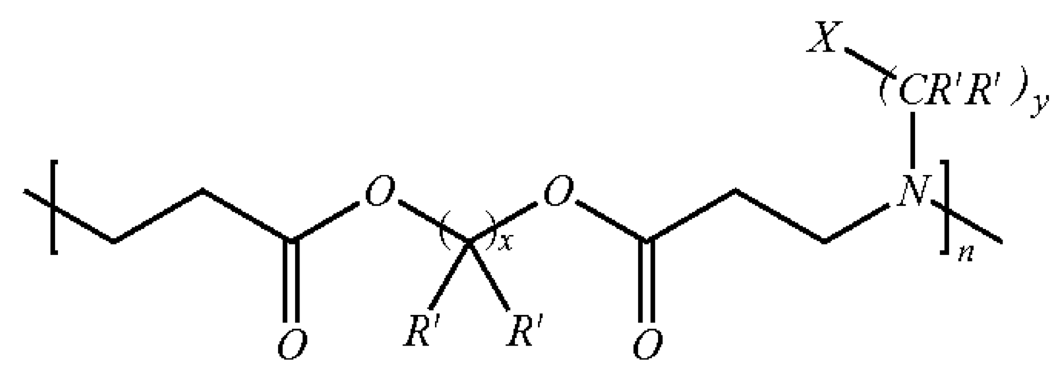
wherein n is an integer between 3 and 10,000; and salts thereof.

**21**. The polymer of claim **1**, wherein the polymer has a molecular weight between 1,000 and 100,000 g/mol.



**22**. The polymer of claim **1**, wherein the polymer has a molecular weight between 2,000 and 40,000 g/mol.

**23**. A pharmaceutical composition comprising a polynucleotide and a polymer of **[**claim **1]** *the formula:*



*wherein X is OR or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

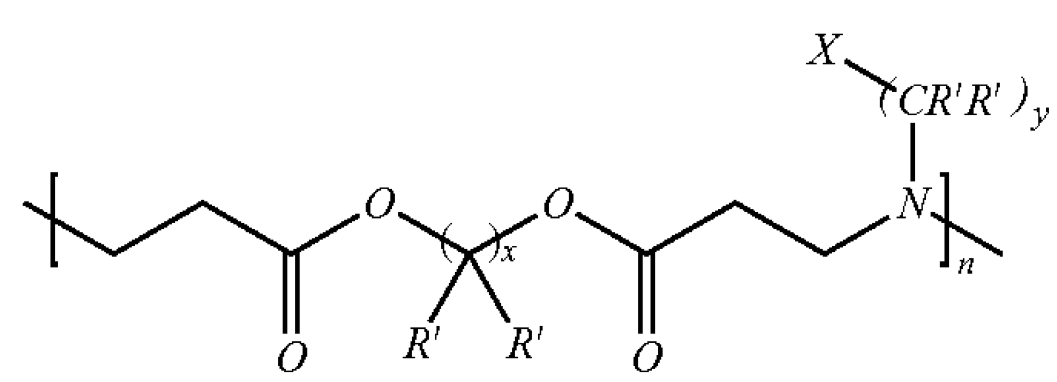
*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 1 and 10; and salts thereof.*

**24**. A pharmaceutical composition comprising nanoparticles containing a polynucleotide and a polymer of **[**claim **1]** *the formula:*



*wherein X is OR or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

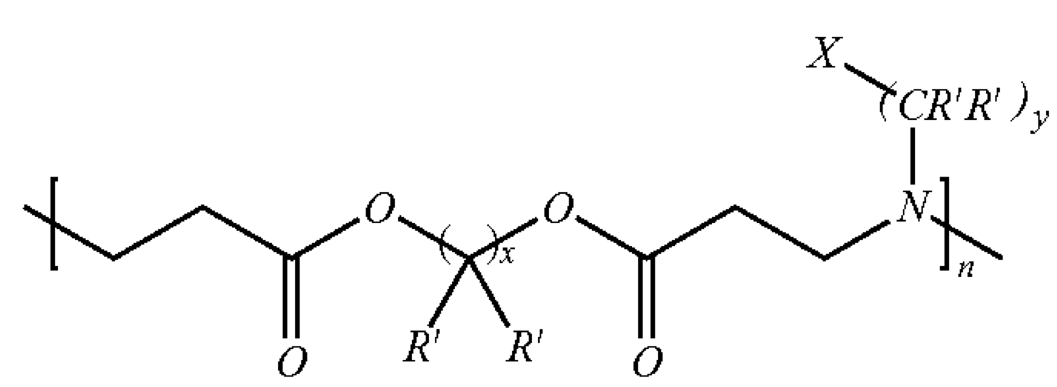
*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 1 and 10; and salts thereof.*

**25**. A pharmaceutical composition comprising nanoparticles containing a pharmaceutical agent and a polymer of **[**claim **1]** *the formula:*



*wherein X is OR or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

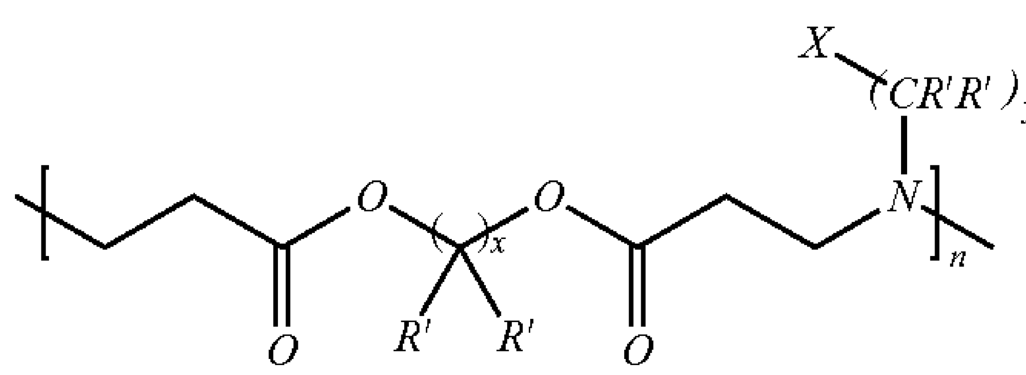
*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy,* *hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 1 and 10; and salts thereof.*

**26**. A pharmaceutical composition comprising microparticles containing pharmaceutical agent encapsulated in a matrix of a polymer of [claim **1**] *the formula:*



*wherein X is OR or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 1 and 10; and salts thereof.*

**27**. The pharmaceutical composition of claim **26** wherein the microparticles have a mean diameter of 1-10 micrometers.

**28**. The pharmaceutical composition of claim **26** wherein the microparticles have a mean diameter of less than 5 micrometers.

**29**. The pharmaceutical composition of claim **26** wherein the microparticles have a mean diameter of less than 1 micrometer.

**30**. The pharmaceutical composition of claim **26** wherein the pharmaceutical agent is a polynucleotide.

**31**. The pharmaceutical composition of claim **30** wherein the polynucleotide is DNA.

**32**. The pharmaceutical composition of claim **30** wherein the polynucleotide is RNA.

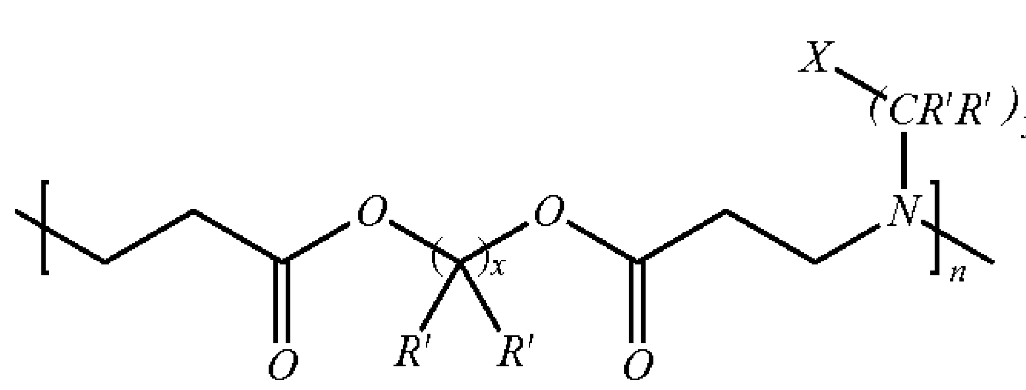
**33**. The pharmaceutical composition of claim **30** wherein the polynucleotide is an siRNA.

**34**. The pharmaceutical composition of claim **26** wherein the pharmaceutical agent is a small molecule.

**35**. The pharmaceutical composition of claim **26** wherein the pharmaceutical agent is a peptide.

**36**. The pharmaceutical composition of claim **26** wherein the pharmaceutical agent is a protein.

*37. A polymer of the formula:*



*wherein X is methyl, OR, or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 6 and 10; and salts thereof.*

*38. The polymer of claim 37, wherein the polymer is amine-terminated.*

*39. The polymer of claim 37, wherein the polymer is acrylate-terminated.*

*40. The polymer of claim 37, wherein x is an integer between 2 and 7.*

*41. The polymer of claim 37, wherein x is 4.*

*42. The polymer of claim 37, wherein x is 5.*

*43. The polymer of claim 37, wherein x is 6.*

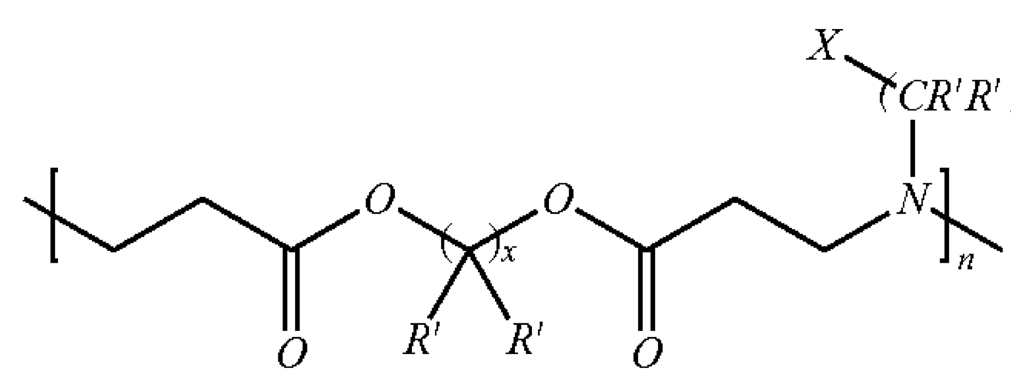
*44. The polymer of claim 37, wherein y is 6.*

*45. The polymer of claim 37, wherein y is 7.*

*46. The polymer of claim 37, wherein R is hydrogen.*

*47. The polymer of claim 37, wherein R is methyl, ethyl, propyl, butyl, pentyl, or hexyl.*

*48. A polymer of the formula:*



*wherein X is methyl, OR, or $NR_2$;*

*R is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, cyclic, heterocyclic, aryl, and heteroaryl;*

*each R' is independently selected from the group consisting of hydrogen, $C_1$-$C_6$ lower alkyl, $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen; wherein at least one R' is independently selected from the group consisting of $C_1$-$C_6$ lower alkoxy, hydroxy, amino, alkylamino, dialkylamino, cyano, thiol, heteroaryl, aryl, phenyl, heterocyclic, carbocyclic, and halogen;*

*n is an integer between 3 and 10,000;*

*x is an integer between 1 and 10;*

*y is an integer between 1 and 10; and salts thereof.*

*49. The polymer of claim 48, wherein the polymer is amine-terminated.*

*50. The polymer of claim 48, wherein the polymer is acrylate-terminated.*

*51. The polymer of claim 48, wherein x is an integer between 2 and 7.*

*52. The polymer of claim 48, wherein x is 4.*

*53. The polymer of claim 48, wherein x is 5.*

*54. The polymer of claim 48, wherein x is 6.*

*55. The polymer of claim 48, wherein y is an integer between 2 and 7.*

*56. The polymer of claim 48, wherein y is 4.*

*57. The polymer of claim 48, wherein y is 5.*

*58. The polymer of claim 48, wherein y is 6.*

*59. The polymer of claim 48, wherein R is hydrogen.*

*60. The polymer of claim 48, wherein R is methyl, ethyl, propyl, butyl, pentyl, or hexyl.*

*61. The polymer of claim 1, wherein X is methyl.*

*62. The polymer of claim 1, wherein X is OR.*

*63. The polymer of claim 1, wherein X is $NR_2$.*

*64. The polymer of claim 37, wherein X is methyl.*

*65. The polymer of claim 37, wherein X is OR.*

*66. The polymer of claim 37, wherein X is $NR_2$.*

*67. The polymer of claim 48, wherein X is methyl.*

*68. The polymer of claim 48, wherein X is OR.*

*69. The polymer of claim 48, wherein X is $NR_2$.*

* * * * *