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BIOMEDICAL AND RELATED APPLICATIONS OF SECOND GENERATION POLYAMIDOAMINES

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Biomedical and Related Applications of Second Generation Polyamidoamines

Content:

1. Synthesis and properties of PAAs
2. PAAs as cytoplasmic delivery vehicles of immunotoxines
3. PAA-cholesterol nanoparticles
4. PAA-β-Cyclodextrin nanoparticles
5. Conclusions

Biomedical and Related Applications of Second Generation Polyamidoamines

1. **Synthesis and properties of PAAs**
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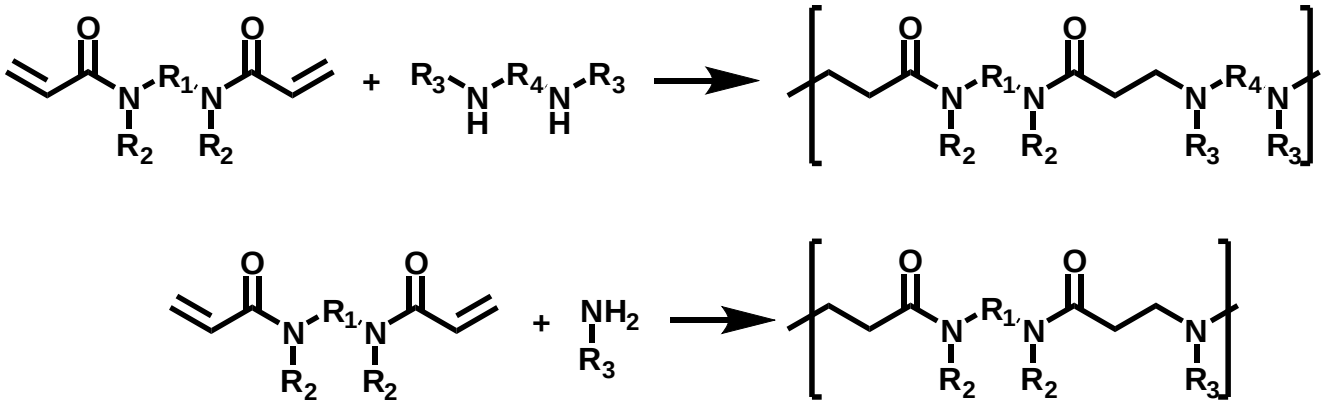
4. PAA-β-Cyclodextrin nanoparticles
5. Conclusions

PAAs: Introduction



Poly(amidoamine)s (PAA) are synthetic polymers containing tert-amine and amide groups regularly arranged along their polymer chain. They are prepared by Michael-type polyaddition of amines to bisacrylamides.

Synthetic conditions:

- room temperature, N_2 atmosphere, 3 days;
- no catalysts;
- protic solvents (water, alcohols).

Franchini, J.; Ferruti, F. *Chapter 16 in Polymeric gene delivery: Principles and Applications.,* Mansoor M. Amiji, CRC Press, 2005.

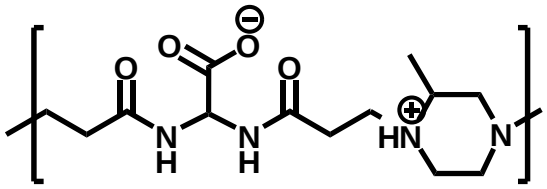
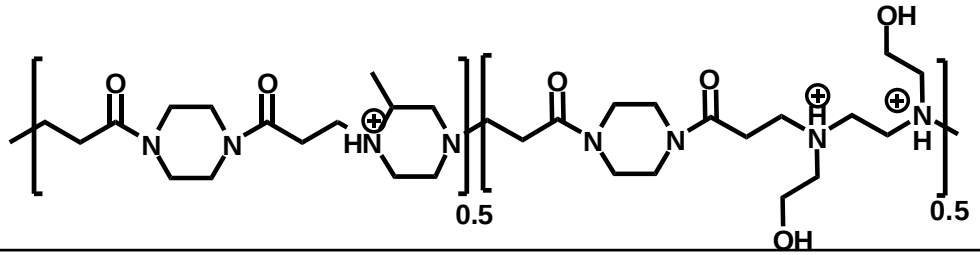
Ferruti, P.; Marchisio, M.A.; Duncan, R *Macromol. Rapid Commun.* **2002**, *23*, 332.

PROPERTIES OF PAAS

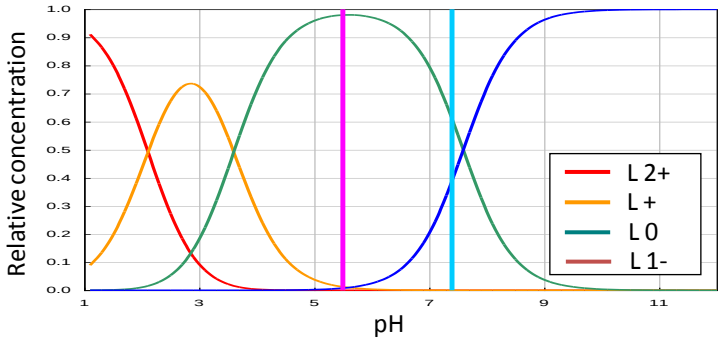
- ✓ PAAs are usually water soluble;
- ✓ PAAs are polymeric bases of low to medium strength;
- ✓ PAAs are biocompatible, biodegradable and non-toxic;
- ✓ Easily functionalized with several different functional groups;
- ✓ Amphoteric PAAs show stealth properties and EPR effect.

Franchini, J.; Ferruti, F. *Chapter 16 in Polymeric gene delivery: Principles and Applications.,* Mansoor M. Amiji, CRC Press, 2005.

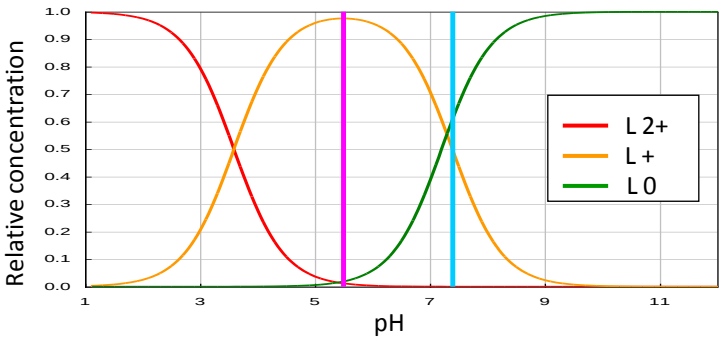
Ferruti, P.; Marchisio, M.A.; Duncan, R *Macromol. Rapid Commun.* **2002**, *23*, 332.

PAA name and structure		pK_a	I.P.	Percentage of Charged Units	
				pH=5.5	pH=7.4
	ISA23	pK_{a1}=2.1 pK_{a2}=3.25 pK_{a3}=7.5	5.5	40% (-)	2% (-)
	ISA1	pK_{a1}=8.1 pK_{a2}=6.9 pK_{a3}=3.8 pK_{a4}=2.8	>10	95% (+)	55% (+)

ISA23



ISA1



Ferruti, P.; Manzoni, S.; Richardson, S.C.W.; Duncan, R.; Pattrick, N.G.; Mendichi, R.; Casolaro, M. *Macromolecules* **2000**, *33*, 7793-7800.

Franchini, J.; Ranucci, E.; Ferruti, P.; Rossi, M.; Cavalli, R. *Biomacromolecules* **2006**, *7* (4), 1215-1222.

Ranucci, E.; Ferruti, P.; Lattanzio, E., Manfredi, M.; Rossi, M.; Mussini, P.R.; Chiellini, F.; Bartoli C. *Journal of Polymer Science: Part A: Polymer Chemistry* **2009**, *4*, 6977.

TOXICITY OF PAAs

Sample	$\overline{Mn}$	IC_{50} (mg/mL) on B16F10 cells
ISA23	21500	> 5.00
ISA1	9500	3.05 ± 0.70
POLY-L-LYSINE	56500	0.05 ± 0.01
DEXTRAN	70000	> 5.00

Cytoxocity test on B16F10 cell line.

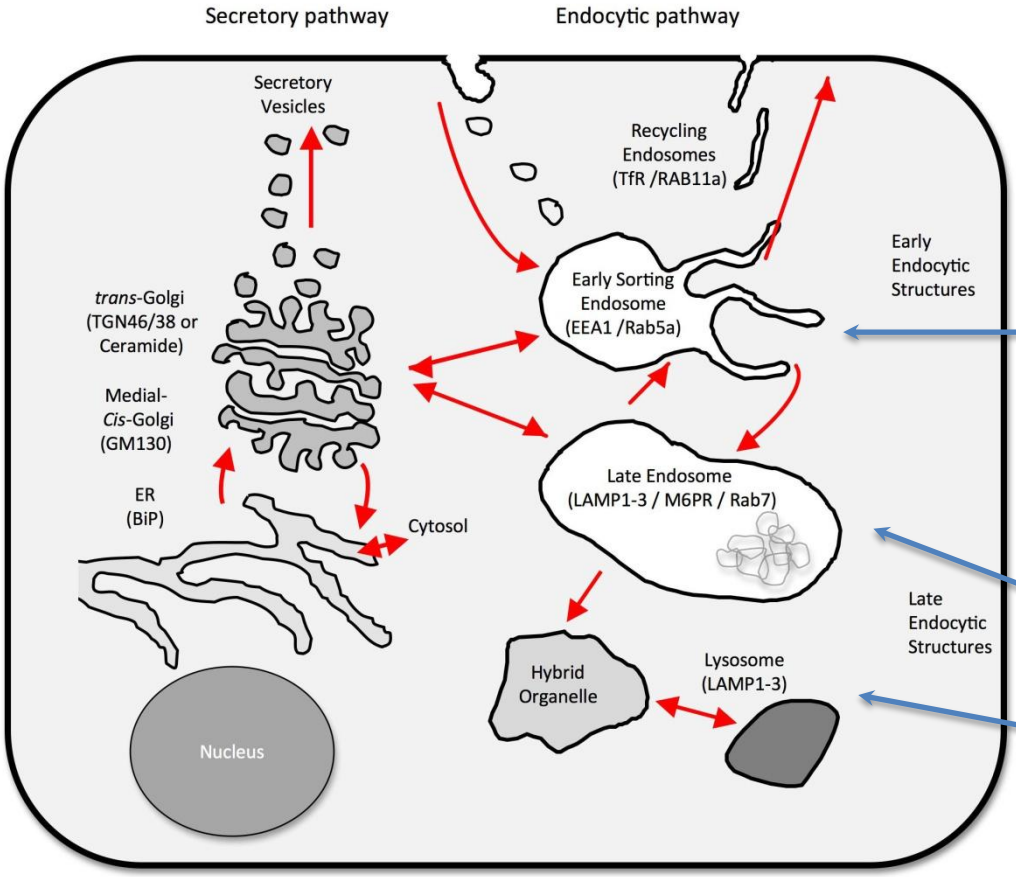
Sample	$\overline{Mn}$	IC_{50} (mg/mL) on Balb/3T3 Clone A31
ISA23	20300	> 5.00
ISA1	14500	2.17 ± 0.75
POLY-L-LYSINE	56500	0.05 ± 0.01
DEXTRAN	70000	> 5.00

Cytoxocity test on mouse embryo fibroblasts Balb/3T3 Clone A31 cell line.

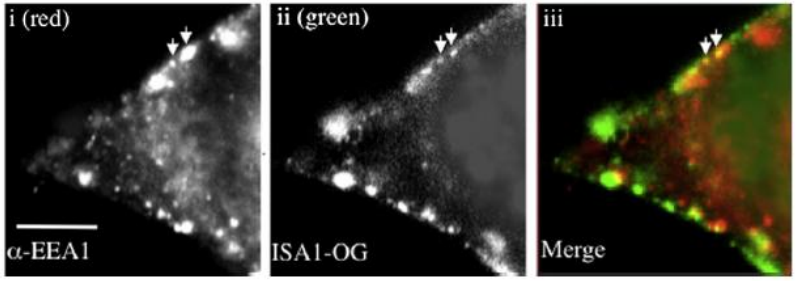
Ferruti, P.; Manzoni, S.; Richardson, S.C.W.; Duncan, R.; Pattrick, N.G.; Mendichi, R.; Casolaro, M. *Macromolecules* **2000**, *33*, 7793-7800.

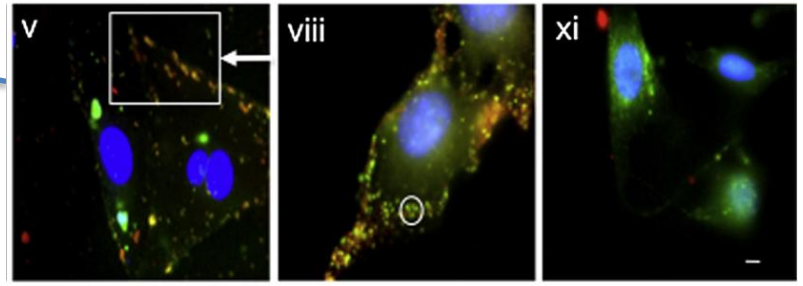
Franchini, J.; Ranucci, E.; Ferruti, P.; Rossi, M.; Cavalli, R. *Biomacromolecules* **2006**, *7* (4), 1215-1222.

Intracellular Trafficking PAAs



Co-localization of ISA1 with EEA1





Co-localization of ISA1 and ISA23 with lysotracker

Richardson, S.C.W.; Pattrick, N.G.; Lavignac, N.; Ferruti, P.; Duncan, R. *J. Contr. Rel.* **2009**, *142* (1), 78-88.

Biomedical and Related Applications of Second Generation Polyamidoamines

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PAAs as Cytoplasmic Delivery Vehicles of Immunotoxines

The development and successful application of therapeutic proteins is often hindered by several difficulties, as for instance insufficient stability and shelf-life, costly production, immunogenic and allergic potential, as well as poor bioavailability and sensitivity towards proteases.

To overcome these problems, a possible approach is to modify proteins by covalently conjugating them with water-soluble polymers, thus increasing their plasma residence, reducing protein immunogenicity and increasing their therapeutic index.

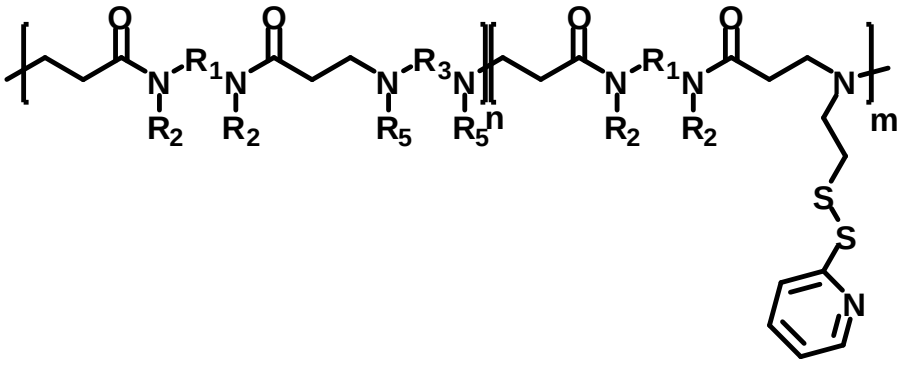
Duncan, R. *Nat. Rev. Drug Discov.* **2003**, *2, 214-221.*

Duncan, R. *Nature Review* **2006**, *6,* 688-701.

Increasing attention has devoted to the nature of the chemical linkage between the polymer backbone and protein.

In this work, two PAAs bearing 2-ethenyldithiopyridine pendants were used to investigate their ability to mediate intracellular delivery of the ribosome-inactivating gelonin.

PAA-SSPy



GELONIN

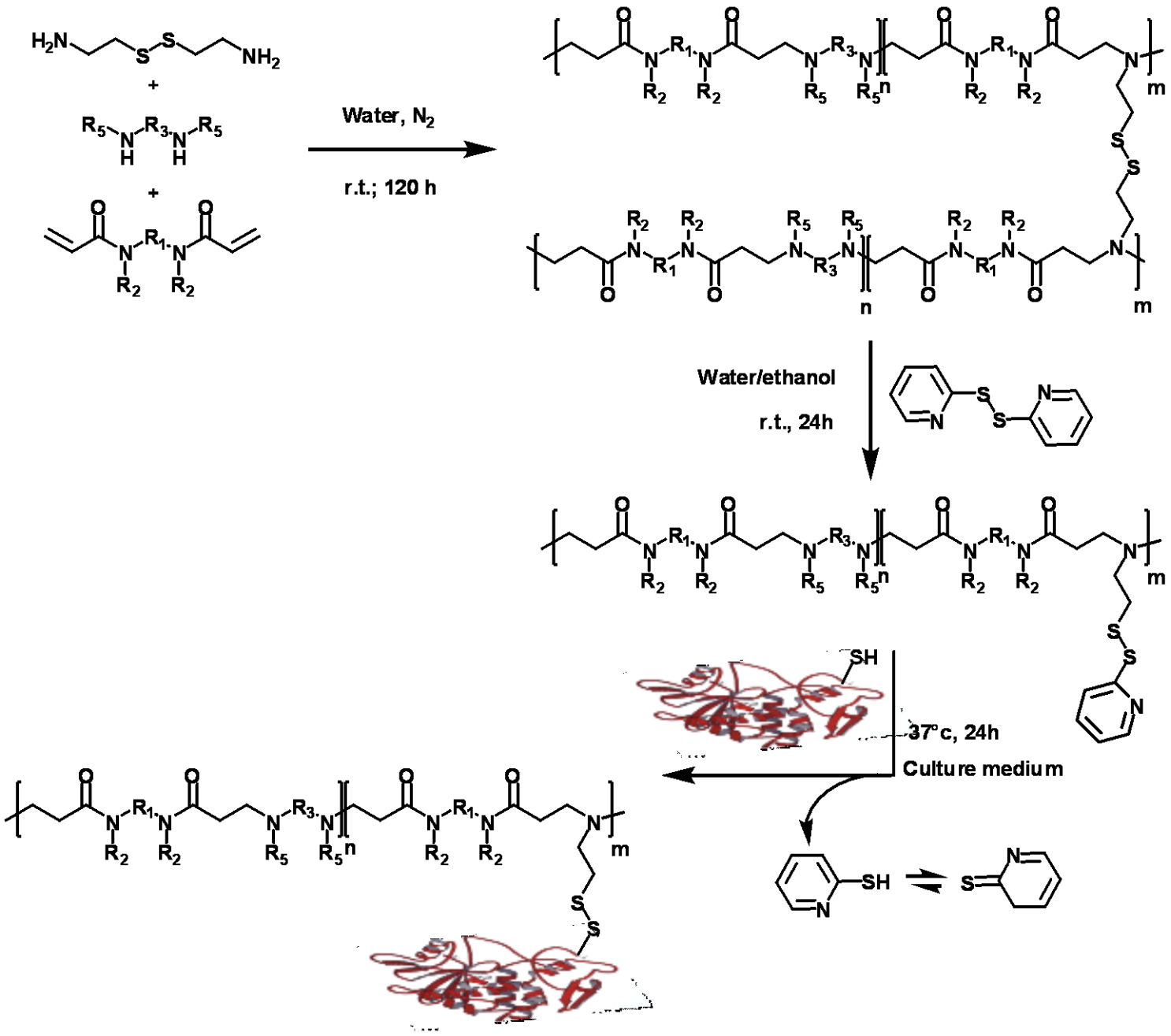
Ribosome-inactivating protein.

It doesn't contain the cell-binding subunit that promotes its internalization by endocytosis.

Emilitri, E.; Ranucci, E.; Ferruti, P. *J. Polym. Sci., Part A: Polym. Chem.* **2005**, *43*, 1404.

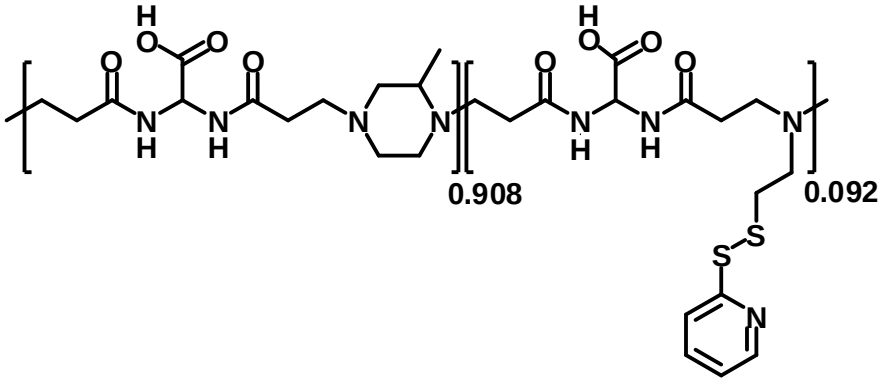
Ranucci, E.; Ferruti, P.; Suardi, M.A.; Manfredi, A. *Macromol. Rapid Commun.* **2007**, *28*, 1243-1250.

PAAs as Cytoplasmic Delivery Vehicles of Immunotoxines

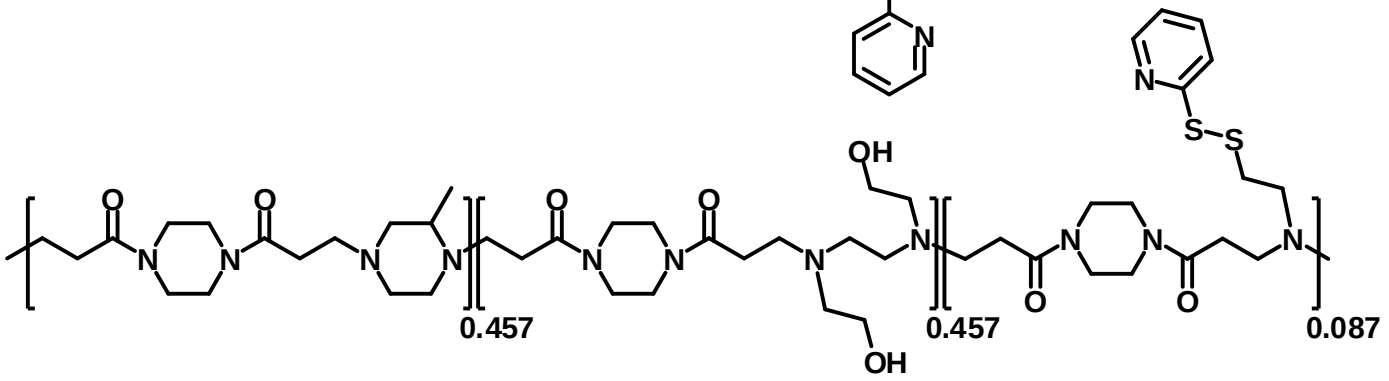


PAAs as Cytoplasmic Delivery Vehicles of Immunotoxines

ISA23-SSPy



ISA1-SSPy



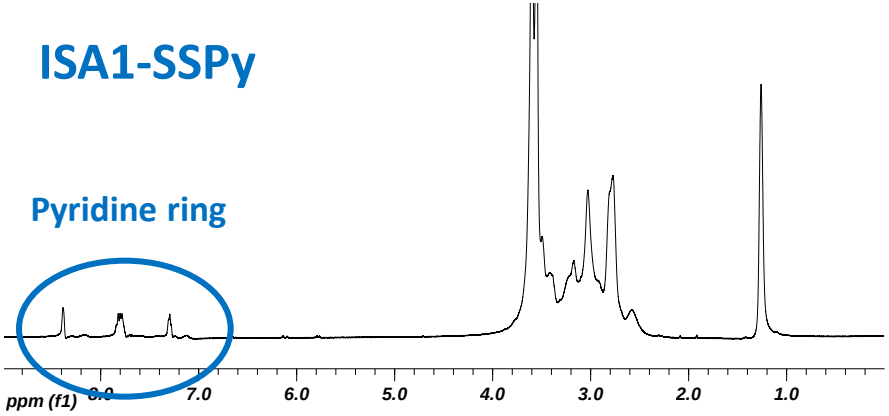
GPC-LS

Sample	$\overline{Mn}$	$\overline{Mw}$	PD	% of functions
ISA1	18000	30000	1.93	8.67
ISA23	12700	23600	1.86	9.2

NMR Spectroscopy

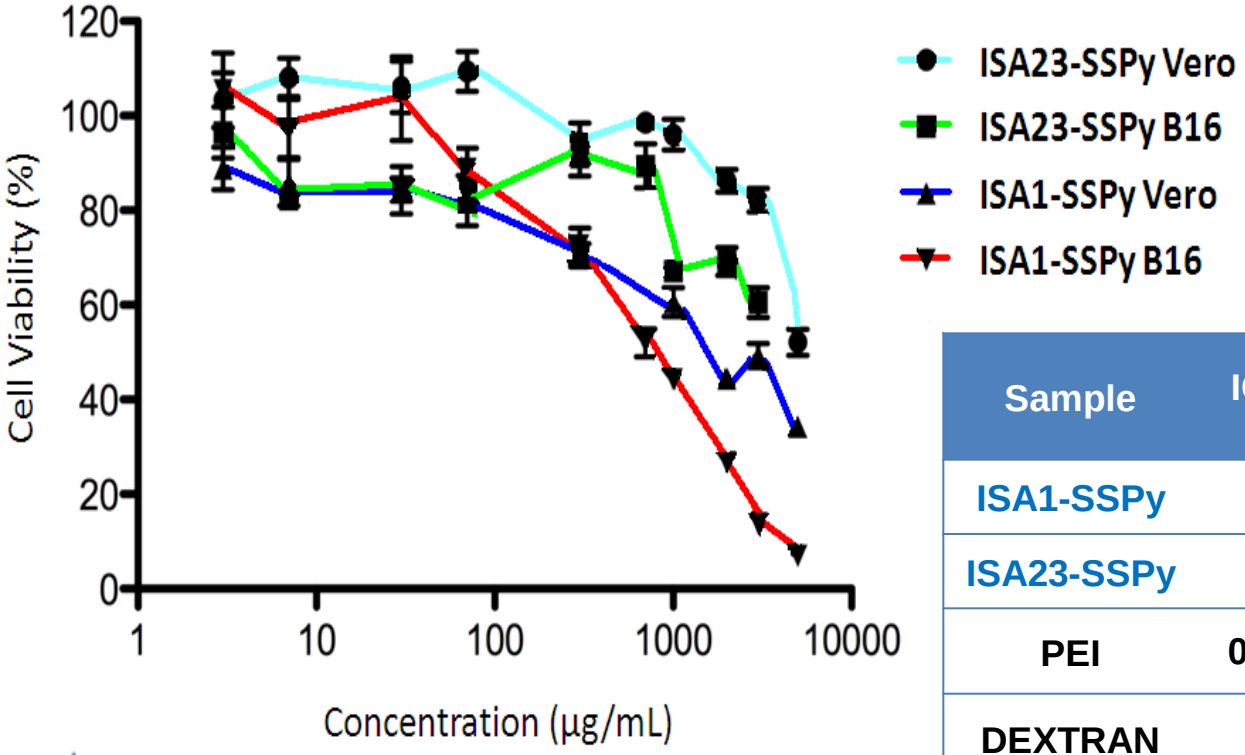
ISA1-SSPy

Pyridine ring



PAA-SSPy Cytotoxicity

ISA1-SSPy and ISA23-SSPy were tested by direct contact assay for 72 hours using B16F10 and Vero cells.



Sample	IC_{50} (mg/mL) on B16F10 cells	IC_{50} (mg/mL) on VERO cells
ISA1-SSPy	2.5	1.5
ISA23-SSPy	> 5.00	5.00
PEI	0.04 ± 0.01	0.05 ± 0.01
DEXTRAN	> 5.00	> 5.00

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Sub-Cloning of Gelonin

PAA-SSPy polymers were used to prepare PAA-gelonin complexes in which the polymeric chain was linked to the bioactive moieties by a disulfide bridge.

To achieve this aim, two types of gelonin were sub-cloned:

6H-V5-Gelonin

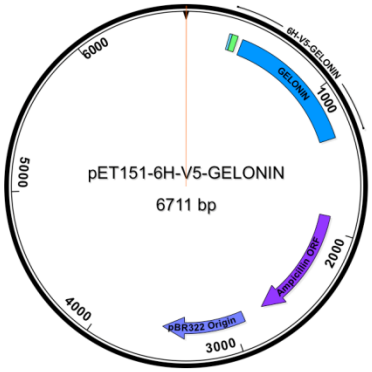
Gelonin-HA-Cys-6H

Plasmids encoding both the two protein were made by sub-cloning an open reading frame coding for gelonin into a commercially available bacterial expression cassette (pET151/D Topo).

Protein were expressed using BL21(DE3) Competent E. Coli.

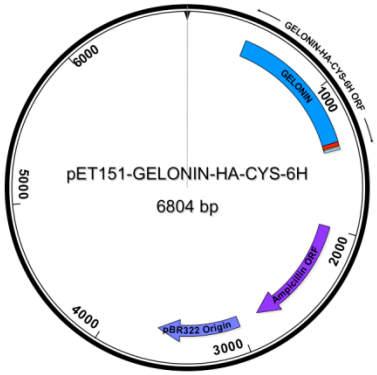
PAAs as Cytoplasmic Delivery Vehicles of Immunotoxines

6H-V5-Gelonin

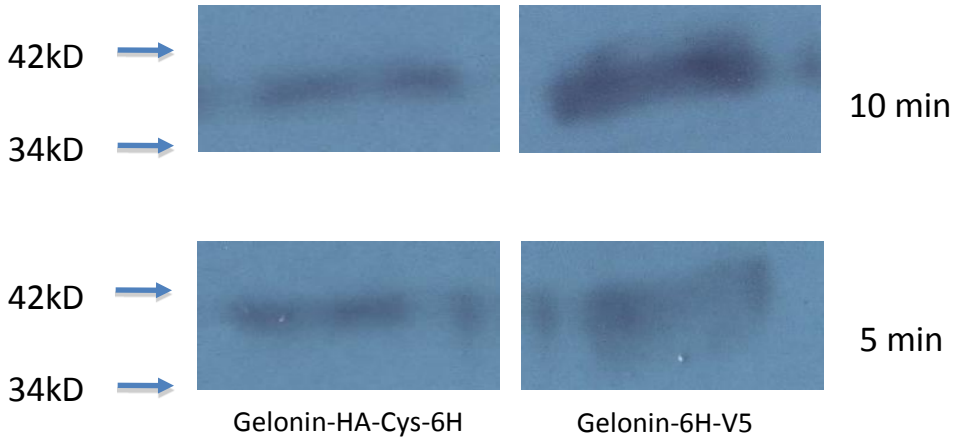


90 **6H** **V5** **Gelonin** 86

Gelonin-HA-Cys-6H



87 **Gelonin** **HA** **6H** 89



1:1000 6XHis Monoclonal Antibody

1:500 Anti-mouse Ig, horseradish whole antibody

Gelonin-HA-Cys-6H

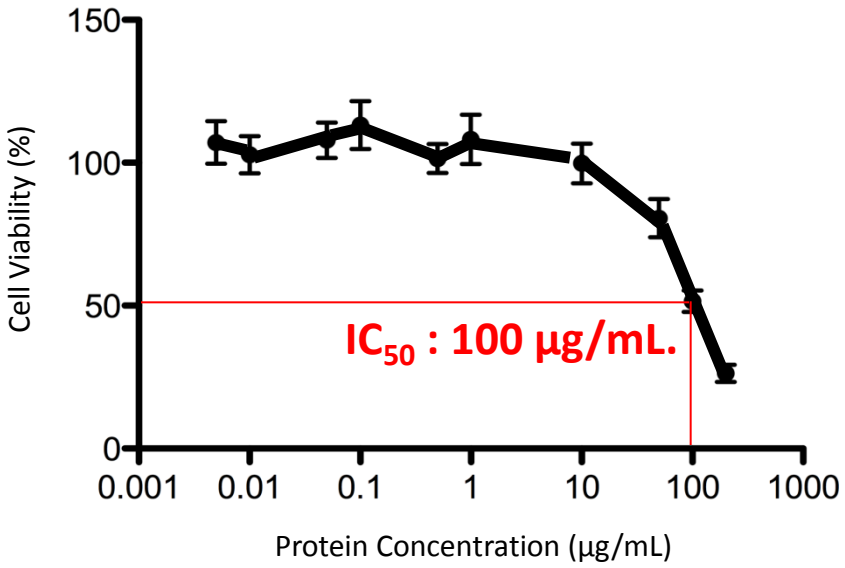
MKGNMKVYWIKIAVATWFCCTTIVL
GSTARIFSLPTNDEEETSKTLGLDTVS
FSTKGATYITYVNFLNELRVKLKPEG
NSHGIPLLRKKCDDPGKCFVLVALSN
DNGQLAEIAIDVTSVYVVGYQVRNR
SYFFKDAPDAAYEGLFKNTIKTRLHF
GGSYPSLEGEKAYRETTDLGIEPLRIGI
KKLDENAIDNYKPTEIASSLLVVIQM
VSEAARFTFIENQIRNNFQQRIRPAN
NTISLENKWGKLSFQIRTSGANGMF
SEAVELERANGKKYYVTAVDQVKPKI
ALLKFVDKDPKTSLAAELIIQNYESLV
GFDESLVGFDYPYDVPDYARCAHHH
HHH.

6H-V5-Gelonin

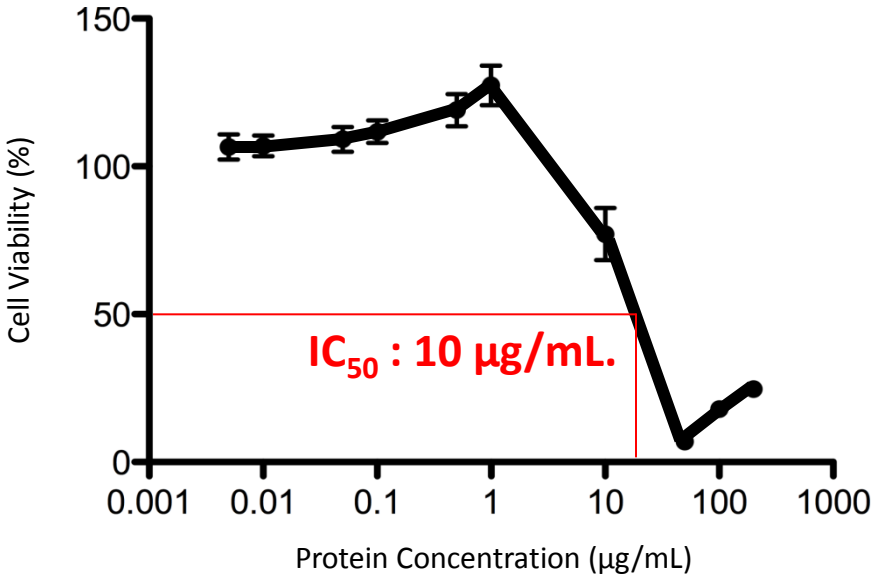
MHHHHHHGKPIPNPLLGLDSTENLYF
QGIDPFTMKGNMKVYWIKIAVATWFC
CTTIVLGSTARIFSLPTNDEEETSKTLGL
DTVSFSTKGATYITYVNFLNELRVKLKPE
GNSHGIPLLRKKCDDPGKCFVLVALSN
DNGQLAEIAIDVTSVYVVGYQVRNRSY
FFKDAPDAAYEGLFKNTIKTRLHFGGSY
PSLEGEKAYRETTDLGIEPLRIGIKKLDE
NAIDNYKPTEIASSLLVVIQMVSEAARF
TFIENQIRNNFQQRIRPANNTISLENK
WGKLSFQIRTSGANGMFSEAVELERA
NGKKYYVTAVDQVKPKIALLKFVDKDP
KTSLAAELIIQNYESLVGFD

Gelonin Toxicity

Gelonin-HA-Cys-6H



6H-V5-Gelonin



In all further experiments, non toxic concentrations of 6H-V5 Gelonin and Gelonin HA-Cys-6H were used.

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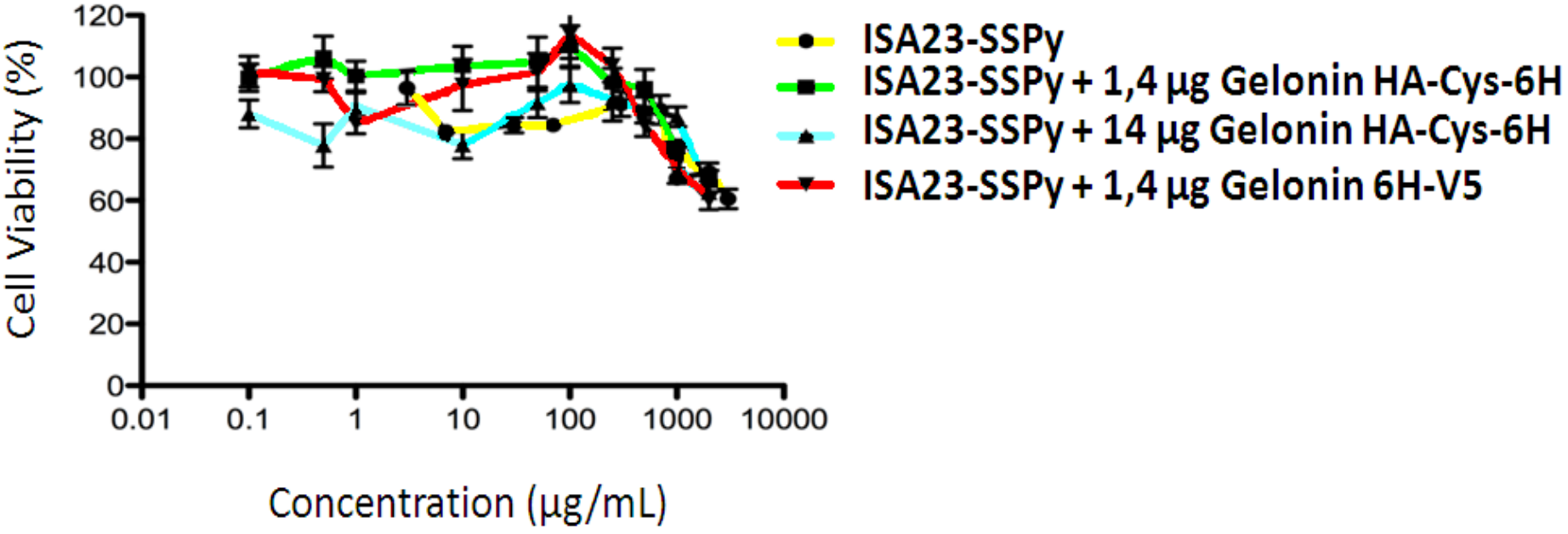
1.4 µg/mL

14 µg/mL

1.4 µg/mL

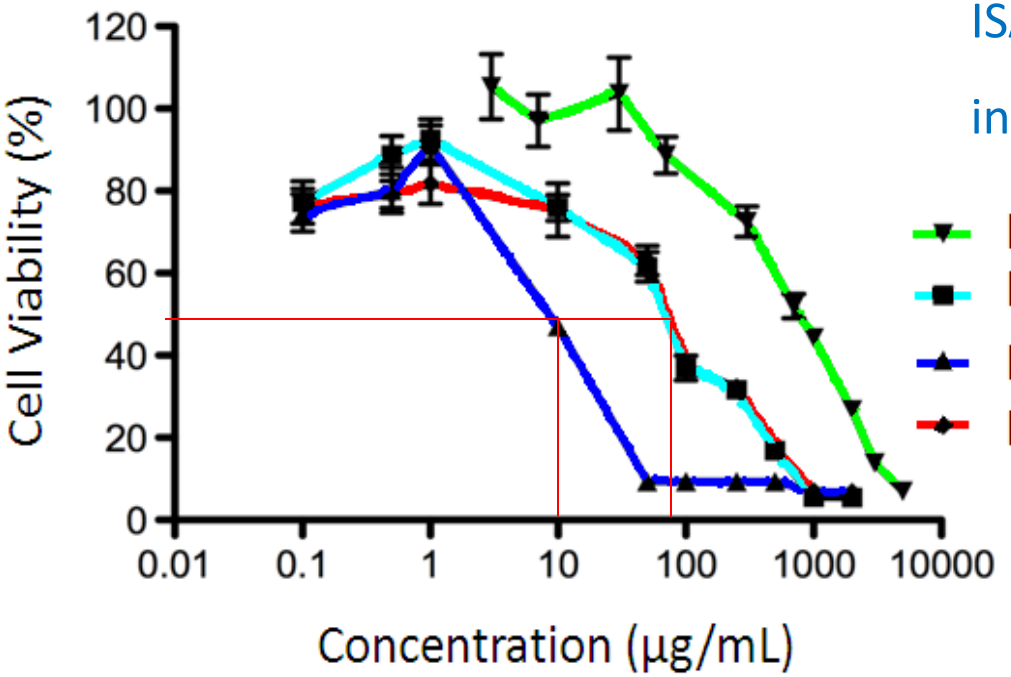
ISA23-SS-Gelonin Conjugates: Toxicity

Cytotoxicity experiments were performed using fixed concentration of protein and polymer concentrations up to 2 mg/mL on B16F10 cells.



ISA23-SSPy was unable to mediate toxin delivery in the B16F10 cells.

ISA1-SS-Gelonin Conjugates: Toxicity



ISA1-SSPy efficiently promoted the intracytoplasmic delivery of gelonin.

ISA1-SSPy-6H-V5 Gelonin →IC_{50} : 100 µg/mL

ISA1-SSPy-Gelonin HA-Cys-V5 →IC_{50} : 100 µg/mL

ISA1-SSPy-Gelonin HA-Cys-6H (14 µg/mL) IC_{50} :10 µg/mL

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PAAs as Cytoplasmic Delivery Vehicles of Immunotoxines

S.C.W. Richardson et al. *Journal of Controlled Release* **2001**, *77*, 225–232

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Journal of Controlled Release 77 (2001) 225–232

journal of controlled release

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Poly(amidoamine)-mediated intracytoplasmic delivery of ricin A-chain and gelonin

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[b]Dipartimento di Scienze e Tecnologie Chimiche e dei Biosistemi, Università di Siena, Piano dei Mantellini 44, 53100 Siena, Italy
[c]Dipartimento di Chimica Organica e Industriale, Universita' degli Studi di Milano, Via Venezian 21, 20133, Milano, Italy

Received 15 May 2001; accepted 7 September 2001

Abstract

Poly(amidoamine)s (PAAs) are water-soluble synthetic polymers designed to be biodegradable and biocompatible. Moreover, they display membrane disruptive properties in response to a decrease in pH. This attribute confers PAAs with endosomolytic properties in vitro and in vivo. A model system was developed to quantify their ability to promote the endosomal escape of macromolecules that may be interesting as therapeutic agents. Here, two PAAs (ISA 1 and 4) were incubated with B16F10 cells in vitro together with two non-permeant toxins: either ricin A-chain (RTA) or gelonin. The relatively non-toxic PAAs ISA 1 and 4 (IC_{50}>1.5 mg/ml) restored activity to the inherently inert toxins. The IC_{50} values for the ISA 1/RTA and ISA 1/gelonin combinations were 0.65±0.05 and 0.55±0.12 mg/ml, respectively. Similarly, when ISA 4 was incubated with a non-toxic combination of RTA and gelonin the IC_{50} value decreased to 0.57±0.03 and 0.43±0.26 mg/ml, respectively. In contrast, the neutral polymer dextran and the PAA ISA 22 were unable to mediate this effect. These observations suggest that specific PAA–toxin combinations warrant further development as novel therapeutics. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Ricin; Gelonin; Poly(amidoamine)s; Non-viral vector; Cytosolic delivery

1. Introduction

Poly(amidoamine)s (PAAs) have been developed as biomedical materials and water soluble drug-carriers, as reviewed in Refs. [1–3]. This family of polymers is currently being developed as a synthetic alternative to fusogenic peptides as they display pH-dependent conformational changes upon protonation at reduced pH which leads to membrane perturbation [4–6]. This property has been shown to confer PAAs with the capacity to promote transfection of pSV-β-galactosidase [6]. Furthermore, PAAs can be designed to be biodegradable, biocompatible and non-hepatotropic [5,7] suggesting their potential for a wide range of in vivo applications.

Plant and bacterial toxins have been widely explored as anticancer agents, particularly in the form of immunotoxins (reviewed in Ref. [8]). Therefore, here we chose to investigate the ability of PAAs to mediate intracellular delivery of two ribosome-inactivating toxins, ricin and gelonin. Ricin, derived from *Ricinus communis* beans [9], is a highly cytotoxic

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E-mail address: duncan@cf.ac.uk (R. Duncan).

PII: S0168-3659(01)00476-X

ISA1-SSPy promoted the intracytoplasmic delivery of gelonin more efficiently than the parent ISA1

ISA1-Gelonin

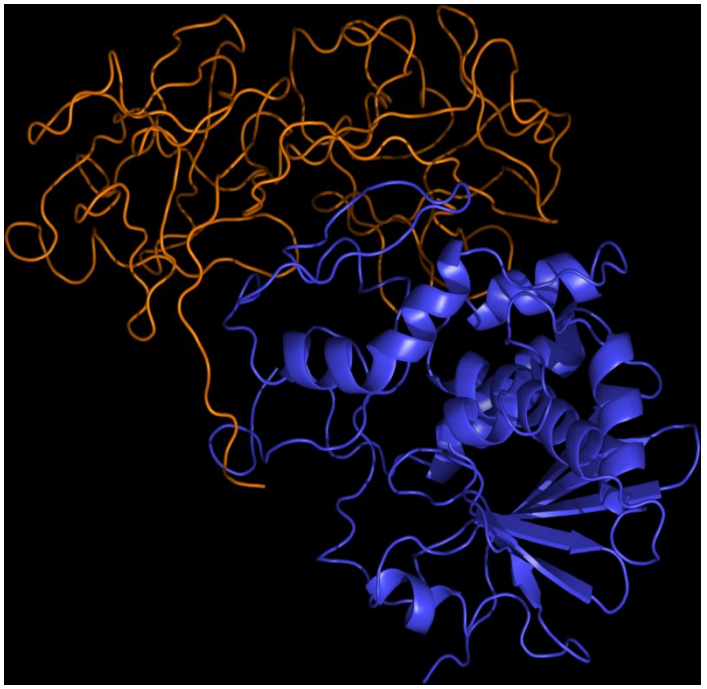
↓

IC_{50} : 522 µg/mL.

ISA1-SSPy-HA-Cys-6H and ISA1-SSPy-6H-V5 Gelonin showed same results (IC_{50}:100 µg/mL).

ISA1-SSPy is able to interact with disulfide groups and hydrophobic domains of the protein, giving stable complexes.

Ricin structure. The **A** chain (RIP) is shown in blue and the **B** chain (cell binding sub-unit) in orange.



ISA1-SSPy acts as synthetic mimicking of the cell binding sub-unit of the Ricin toxin that mediates the internalization of gelonin into the cytosol.

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PAA-CHOLESTEROL NANOPARTICLES

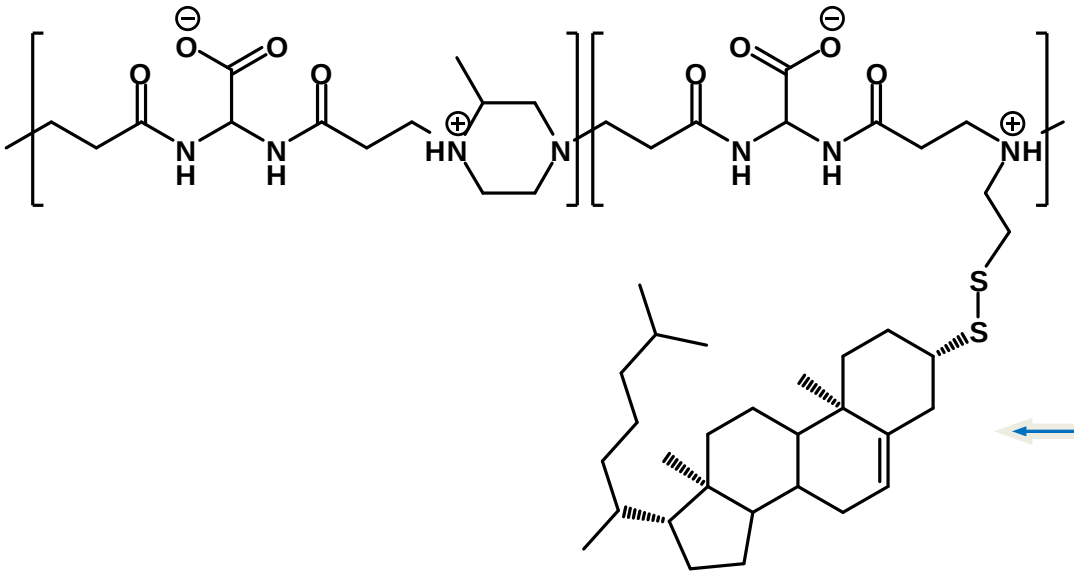
Improvement in drug controlled release is one of the main challenges of modern pharmacology in order to reduce side effects of therapies and to exploit drug potential:

- ✓ "Solubilization" of lipophilic drugs;
- ✓ Specific targeting and release of drugs;
- ✓ Sustained release of soluble/insoluble drugs;
- ✓ Protection of proteins or genes in transfection.

The use of polymers in designing new drugs architectures, most often in the form of nanoparticles (NP) is one of the most promising possibilities to achieve these goals.

PAA-CHOLESTEROL NANOPARTICLES

Using the PAA-SSPy precursors already shown, a family of amphoteric polymers containing cholesterol pendants linked through disulfide bonds was synthesized.

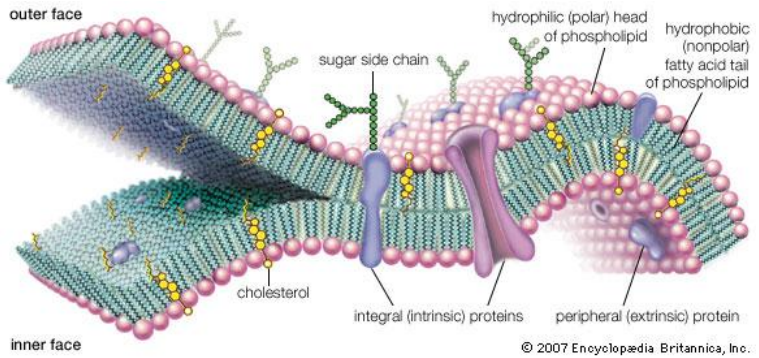


ISA23 backbone

Stealth properties

IC_{50}= 5 mg/ml (B16 cells)

Cholesterol pendants



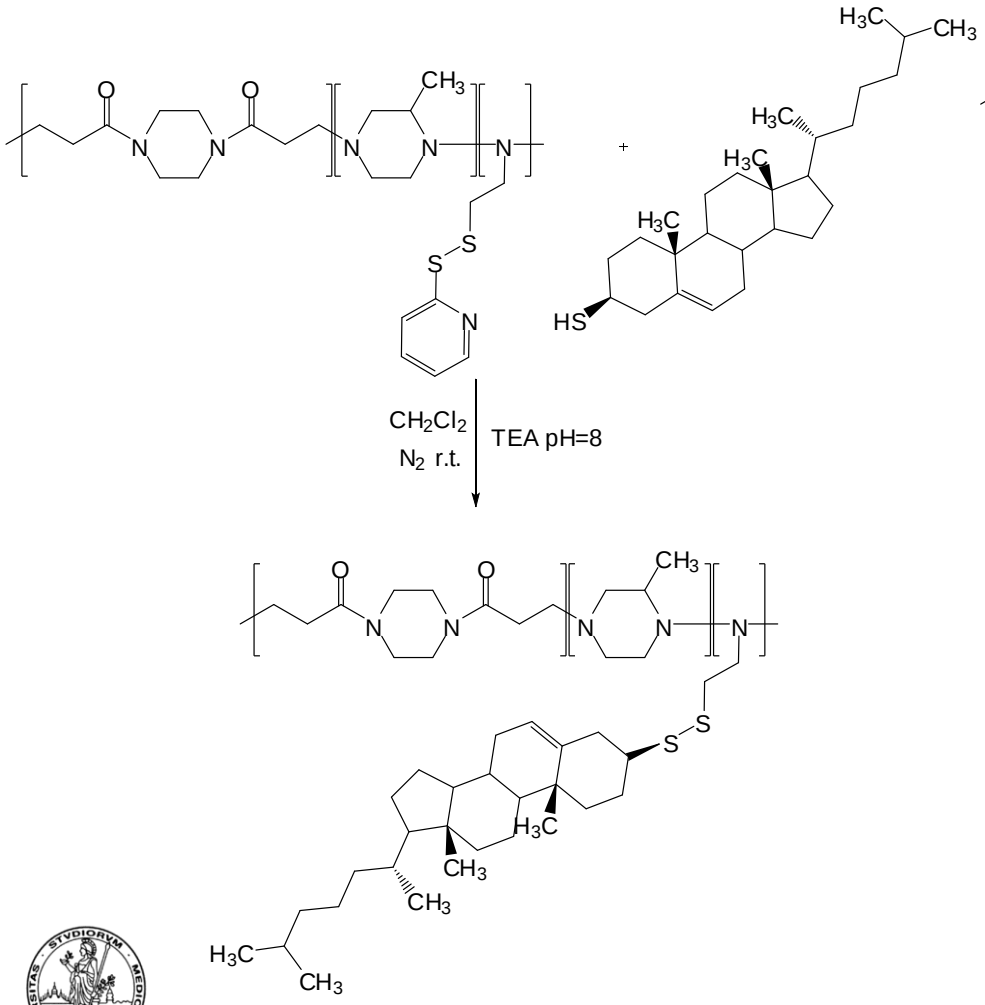
Choletserol is an essential structural component of mammalian cell membranes, where it is required to establish proper membrane permeability and fluidity.

PAA-cholesterol conjugates are able to spontaneously self assemble in nanoparticles.

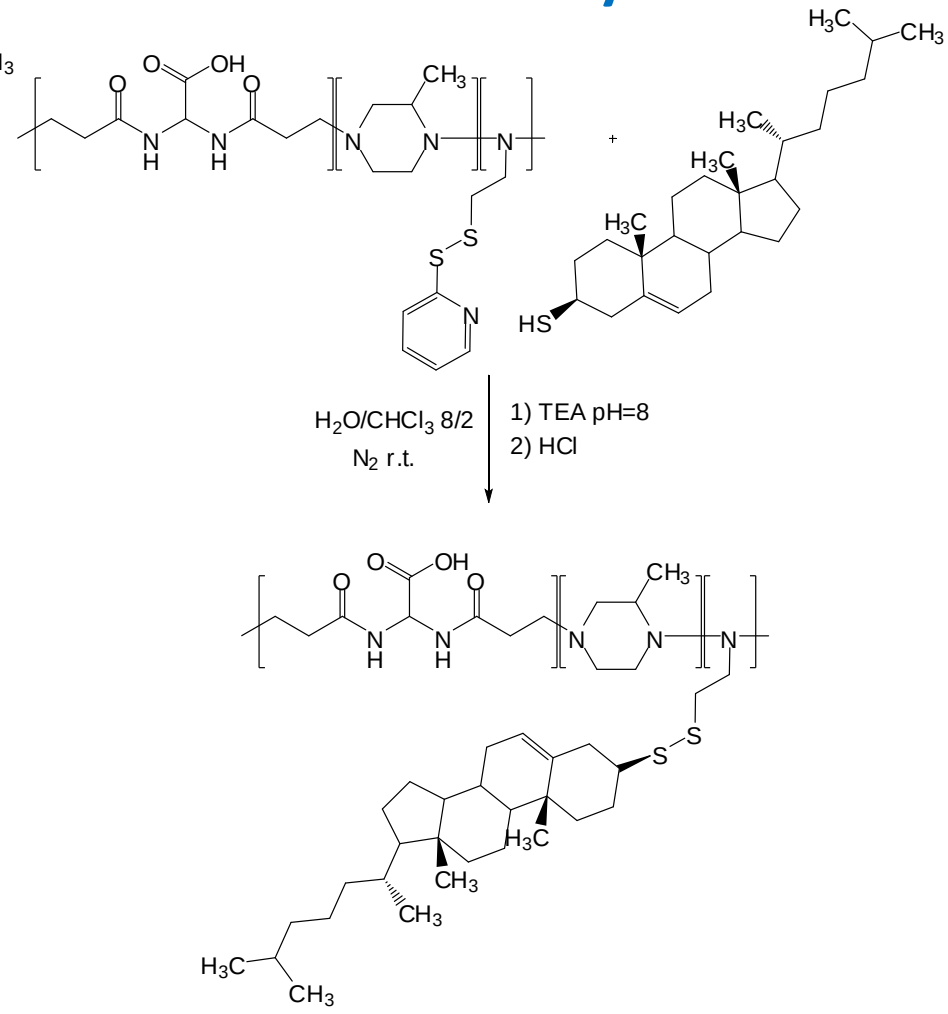
Yusa, S. I.; Kamachi, M.; Morishima, Y. *Macromolecules* **2000,** 33, 1224–1231.
Xu, J. P.; Ji, J.; Chen, W. D.; Shen, J. C. *Macromol. Biosci.* **2005**, 5, 164–171.

PAA-ss-Cholesterol Conjugates: Synthesis

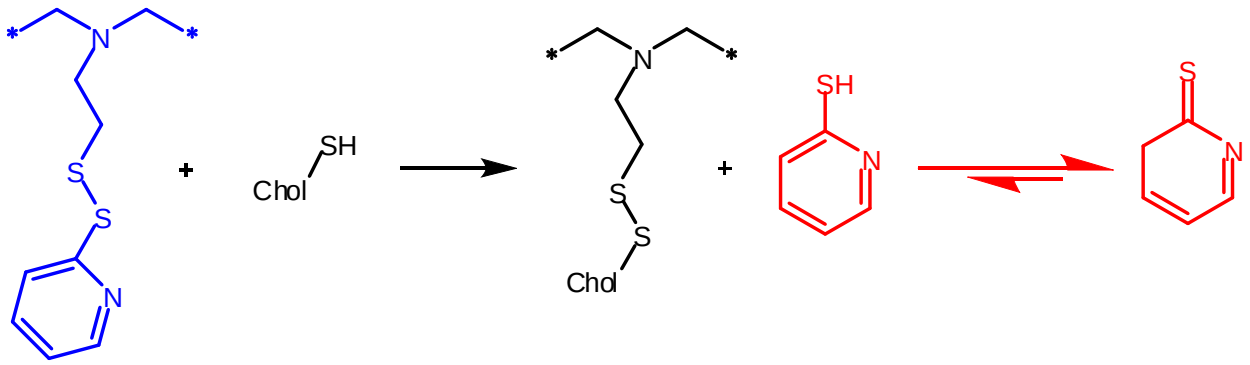
ISA1-SSPy

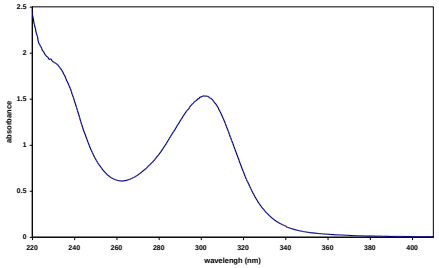


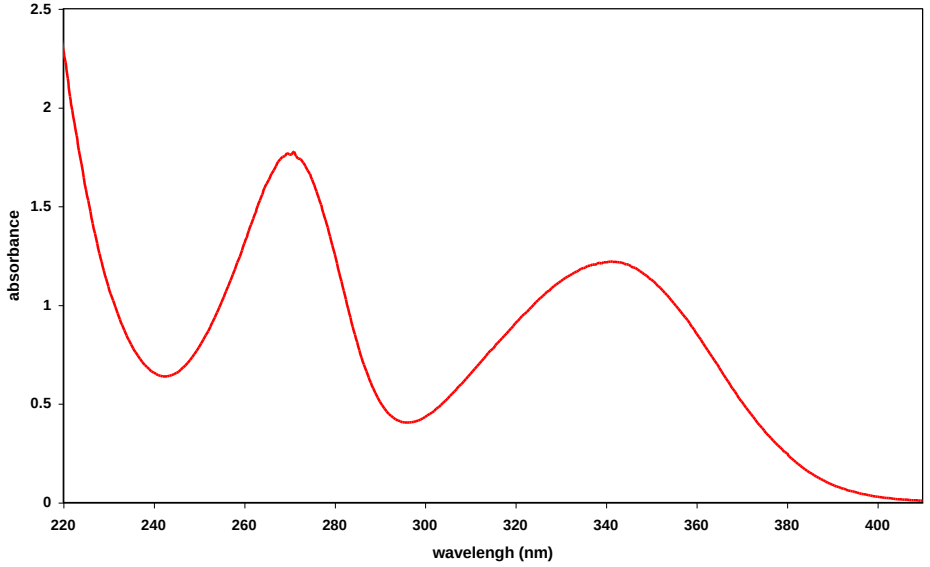
ISA23-SSPy

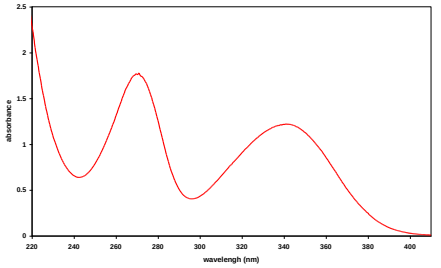


PAA-ss-Cholesterol Conjugates: Synthesis



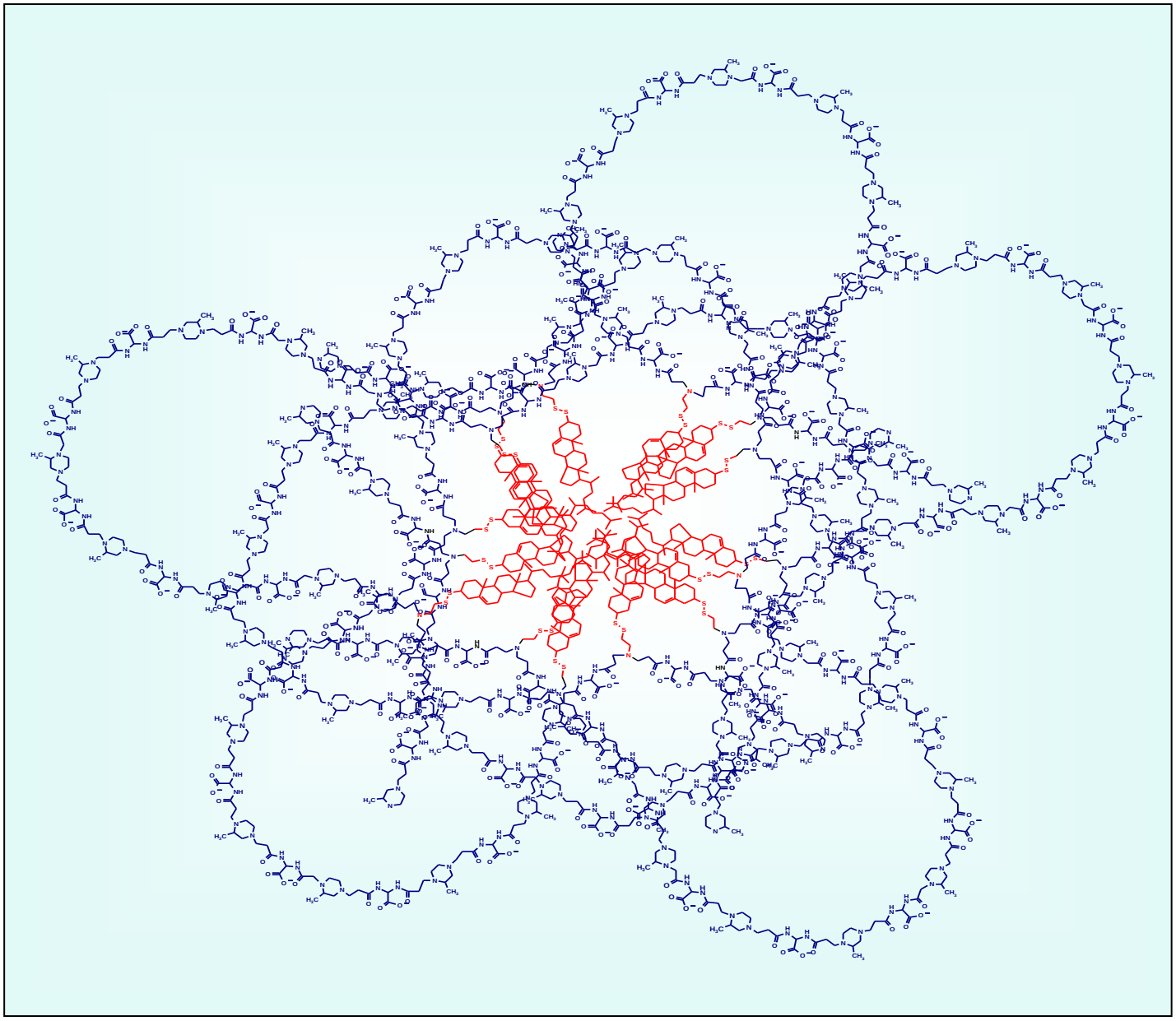






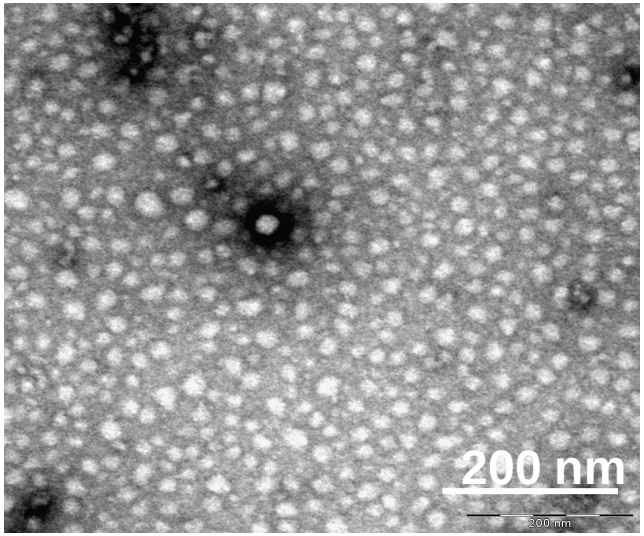
Ranucci, E.; Ferruti, P.; Suardi, M.; Manfredi, A. *Macromol. Rapid. Commun.*, **2007**, 28, 1243

PAA-CHOLESTEROL NANOPARTICLES



SPONTANEOUSLY ASSEMBLED PAA-CHOLESTEROL NP

TEM MICROGRAPH AND DLS



Sample	% Cholesterol	D (nm)	PI
ISA1-SSChol1	8	243 ± 16	0.20
ISA1-SSChol2	15	264 ± 21	0.18
ISA23-SSChol1	8	124 ± 6	0.11
ISA23-SSChol2	15	131 ± 7	0.13

D = average diameter.
PI = polydispersity index.

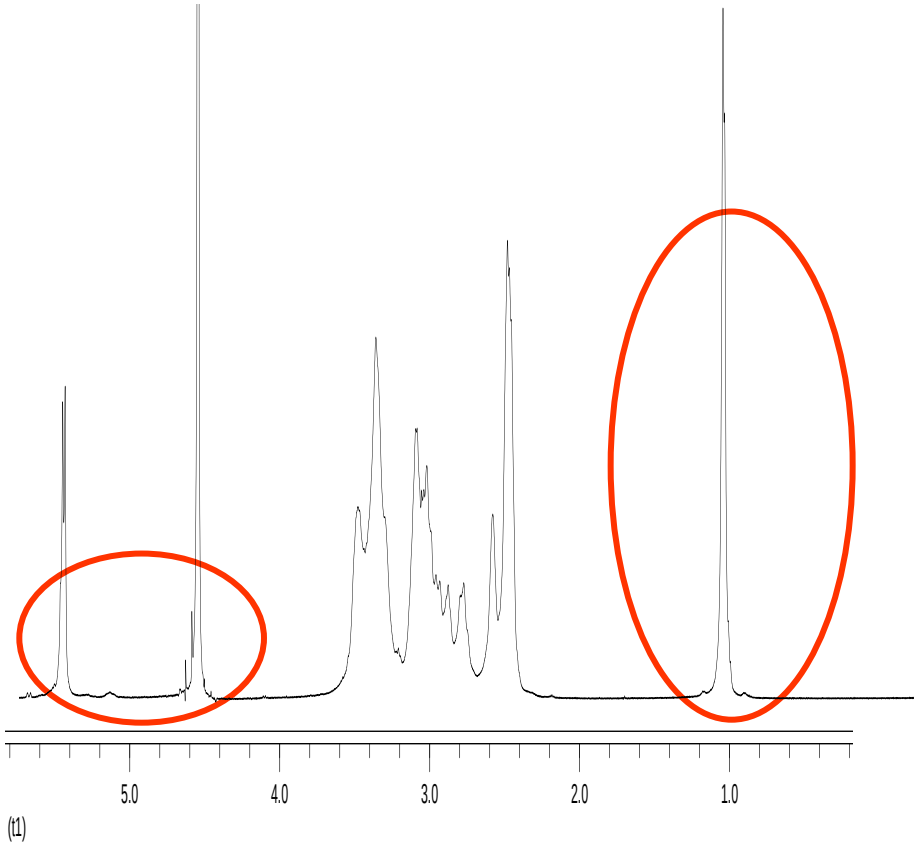
CYTOTOXICITY

Sample	IC_{50} (mg/mL) on 3T3/BALB-c cells
ISA1-SSChol	> 2
ISA23-SSChol	> 3

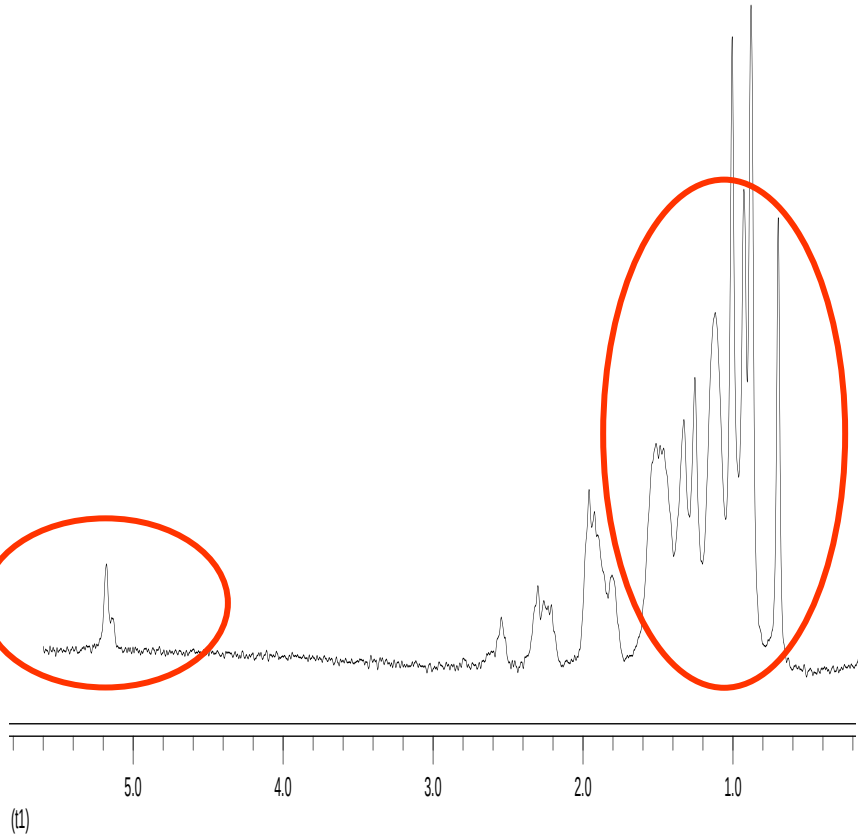
The cytotoxicity of PAA-cholesterol conjugates was assessed by in vitro cytotoxicity assays performed against 3T3/BALB-c Clone A31 cell lines

^{1}H NMR OF PAA-CHOLESTEROL CONJUGATES

NMR spectrum in D_2O

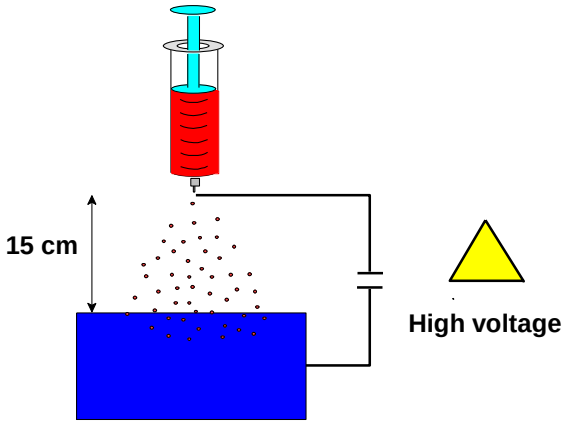


NMR spectrum in $CDCl_3$



Ranucci, E.; Suardi, M. A.; Annunziata, R.; Ferruti, P.; Chiellini, F.; Bartoli,C. *Biomacromolecules,* **2008**, 9 (10), 2693-2704

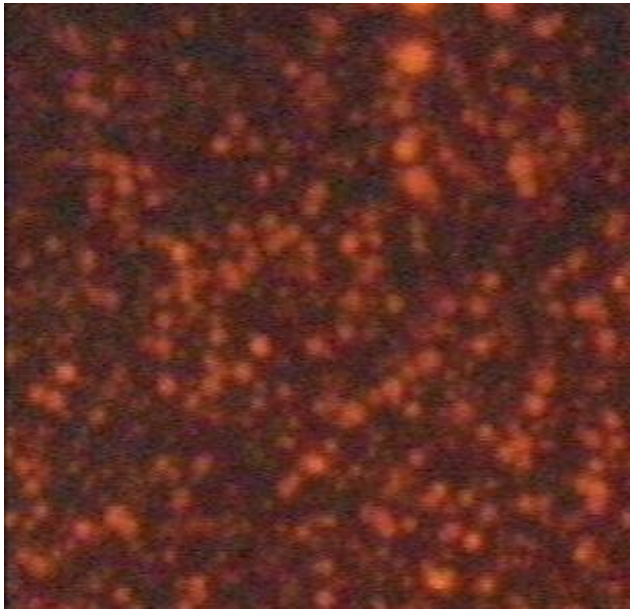
FORMULATION OF DRUG LOADEAD PAA-CHOLESTEROL NP



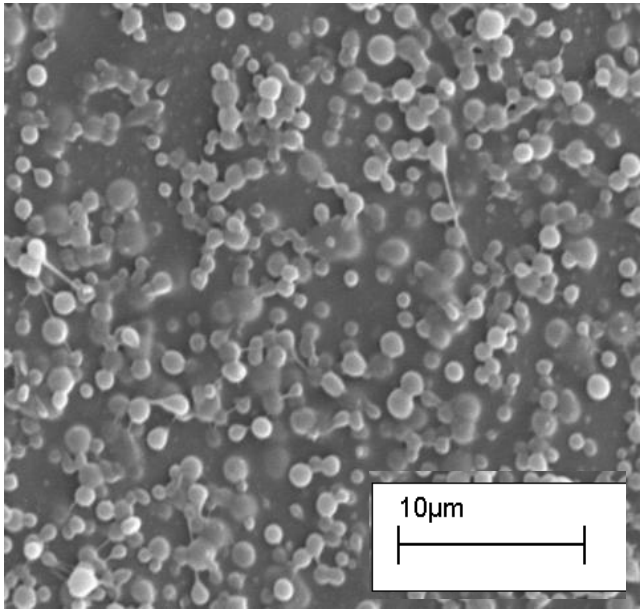
ELECTROSPRAY

Electrospray is a method of liquid atomization that consists in the dispersion of a solution into small charged droplets by an electric field.

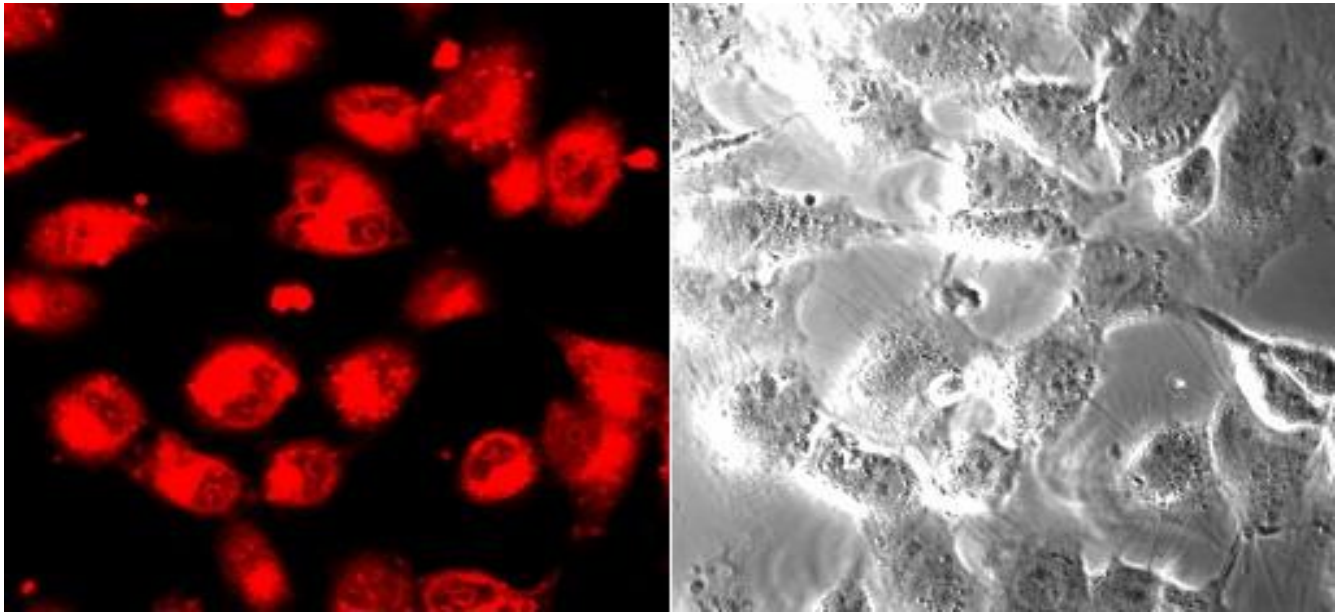
Doxorubicin loaded (9%) PAA-cholesterol nanoparticles



Tamoxifen loaded (5%) PAA-cholesterol nanoparticles



FORMULATION OF DRUG LOADEAD PAA-CHOLESTEROL NP ELECTROSPRAY



Cellular uptake of doxorubicin-loaded and fluorescent PAA-cholesterol nanoparticles with confocal laser scanning microscopy.

After 1 hour the nanoparticles were internalized in the cells.

FORMULATION OF DRUG LOADEAD PAA-CHOLESTEROL NP SOLVENT INJECTION METHOD

PAA-cholesterol nanoparticles were formulated by the solvent injection method from water-ethanol mixtures.

The nanoparticles showed no hemolytic activity tested on in vitro red blood cell.

Sample	D (nm)	PI	PZ (mV)
ISA23-SSChol	60 ± 10	0.26	-14.86 ± 0.99

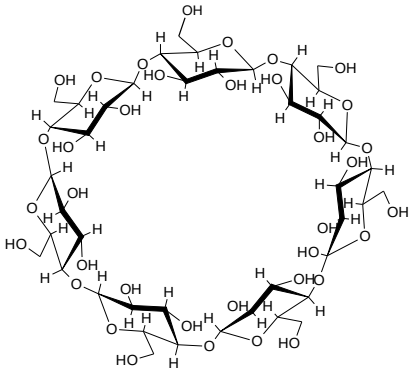
D = average diameter.
PI = polydispersity index.
PZ = zeta potential

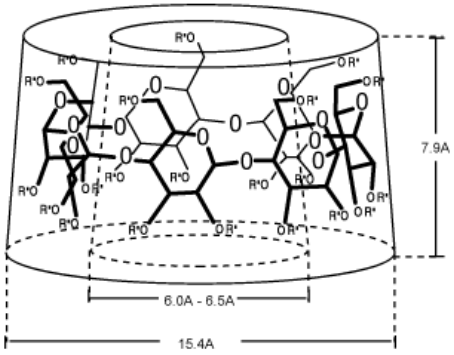
Biomedical and Related Applications of Second Generation Polyamidoamines

1. Synthesis and properties of PAAs
2. PAAs as cytoplasmic delivery vehicles of immunotoxines
3. PAA-cholesterol nanoparticles
4. **PAA-β-Cyclodextrin nanoparticles**
5. Conclusions

PAA-β-CYCLODEXTRIN NANOPARTICLES

Unsubstituted β-cyclodextrin (β-CD) is poorly soluble in water and often gives nearly insoluble complexes with hydrophobic molecules.





Characteristics	β-CD
Units	7
PM	1135
Water solubility	1.85 (g/100 mL)
Cavity volume	262 Å
$[\alpha]_D$ 25°c	162.5 + 0.5
pK_a	12.2

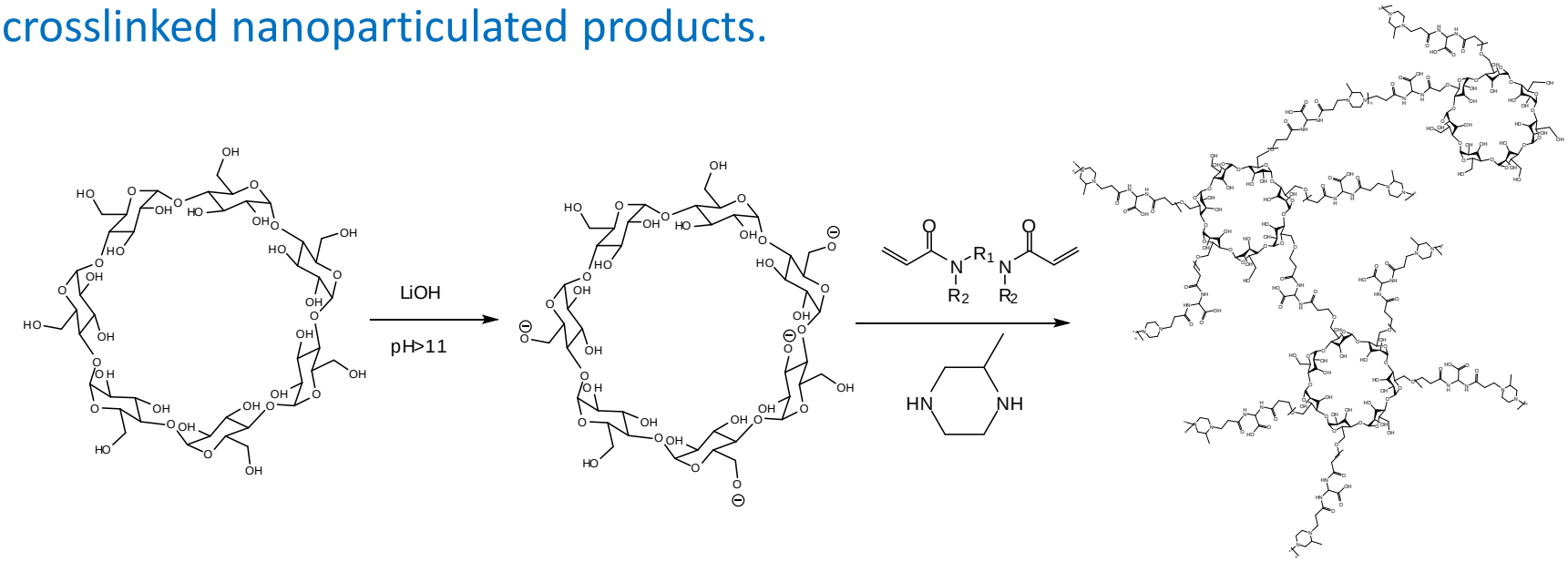
Many chemical modifications have been proposed for increasing the solubility of β-CD and its complexes in aqueous media but only relatively few examples of β-CD conjugates with biocompatible hydrophilic synthetic polymers can be found in literature.

Szejtli, J. *Chem.Rev.* **1998**, 98, 1743.

Salmaso, S.; Semenzato, A.; Calicetti, P.; Hoebeke, J.; Sonico, F.; Dubernet, C.; Couvreur, P. *Bioconjugate Chem.* **2004**, 15, 997.

PAA-β-CYCLODEXTRIN NANOPARTICLES

β-CD-PAA copolymers can be obtained as hyperbranched soluble products, or as crosslinked nanoparticulated products.



In water at 20-25°C and pH ≥ 11, approximately 5 hydroxyl groups per β-CD molecule undergo Michael-type addition to bis-acrylamides.

This means that β-CD acts as multifunctional monomer in stepwise polyadditions to bisacrylamides.

SYNTHETIC STRATEGIES

FLORY-STOCKMAYER EQUATION

Kinetic control	Stoichiometric control
$p_c = \frac{1}{\{r[1+\rho(f-2)]\}^{1/2}}$	$r_c = \frac{1}{1+\rho\,(f-2)}$

r = ratio between the number of functions of the multifunctional monomer and the total number of the same functions

f = number of functions of the multifunctional monomer

r = starting ratio among the complementary functions (“a” and “b”)

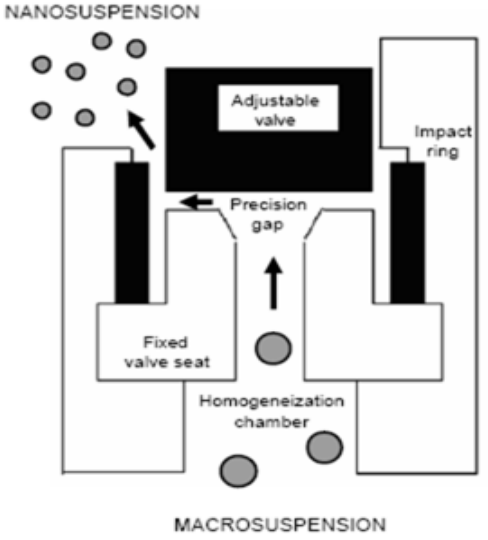
p_c = reaction conversion degree at which gelling takes place;

r_c = critical functiona ratio. Below this value cross-linking cannot take place.

PAA-β-CD NANOPARTICLES

HIGH PRESSURE HOMOGENIZATION (HPH)

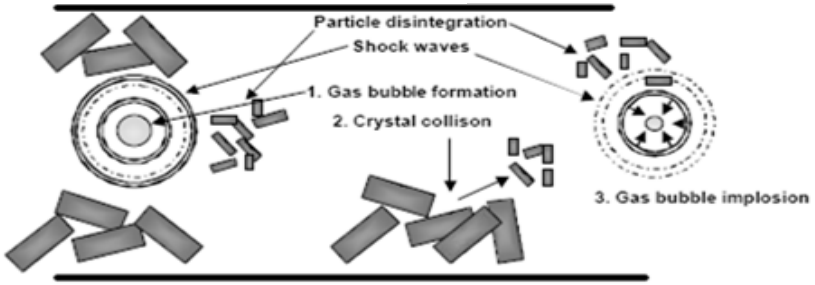
High pressure homogenizer



PAA micro- and nanogels are obtained by high pressure homogeneization of hydrogel suspensions.

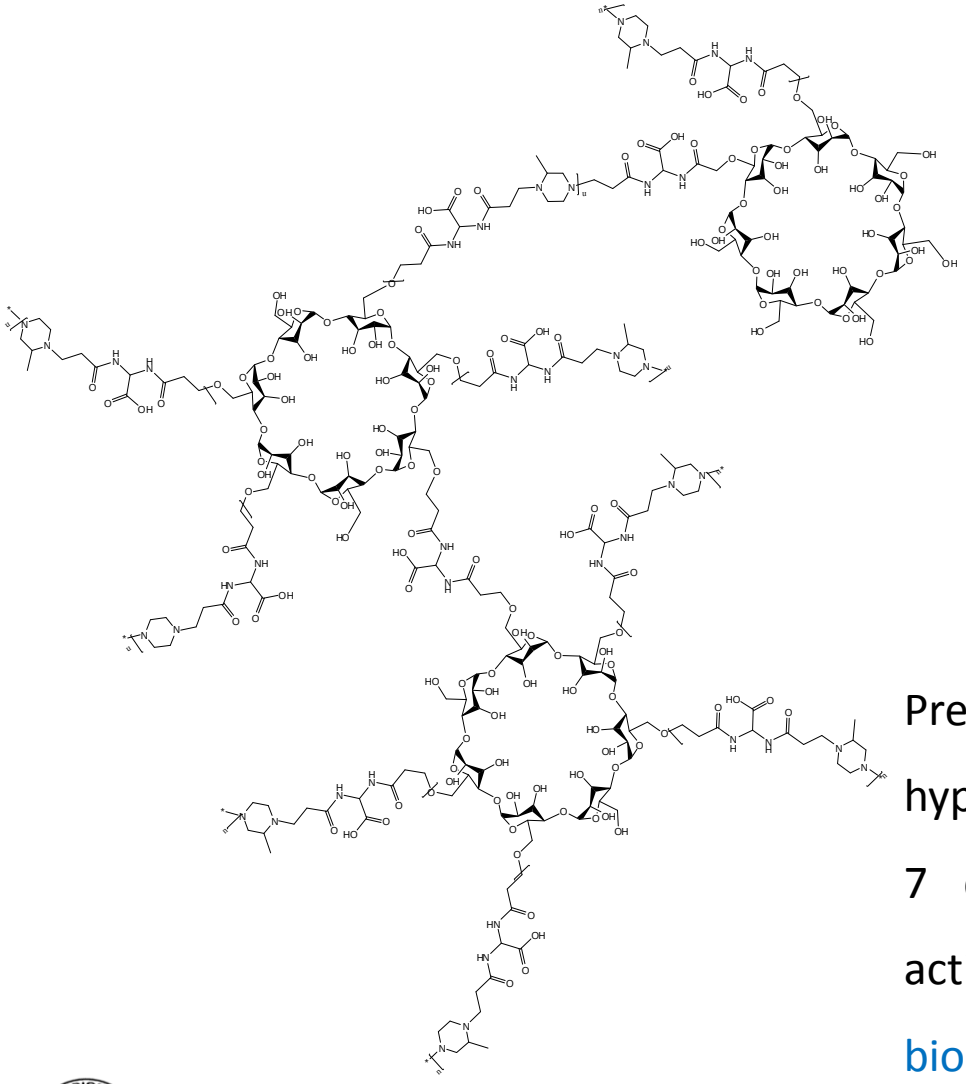
Procedure:

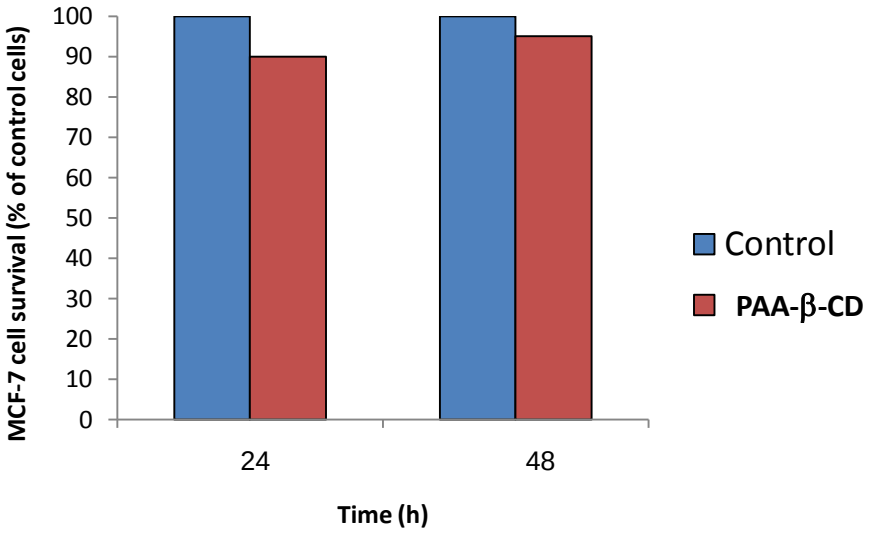
1) mechanical grinding at 12000 rpm
2) Homogeneization cycles at 2000 – 5000 KPa



Particles rupture in precision gap during HPH process

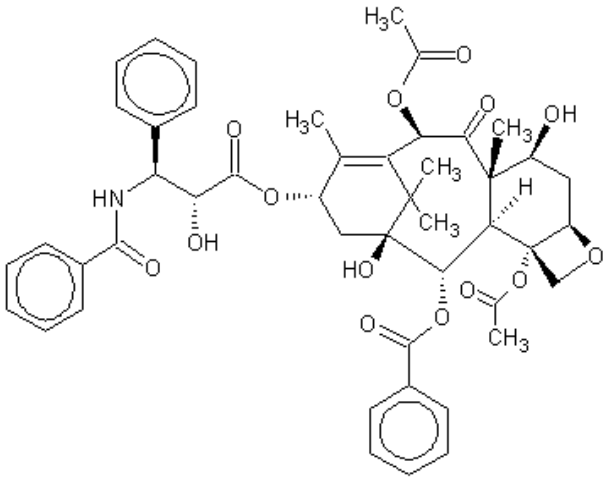
TOXICITY



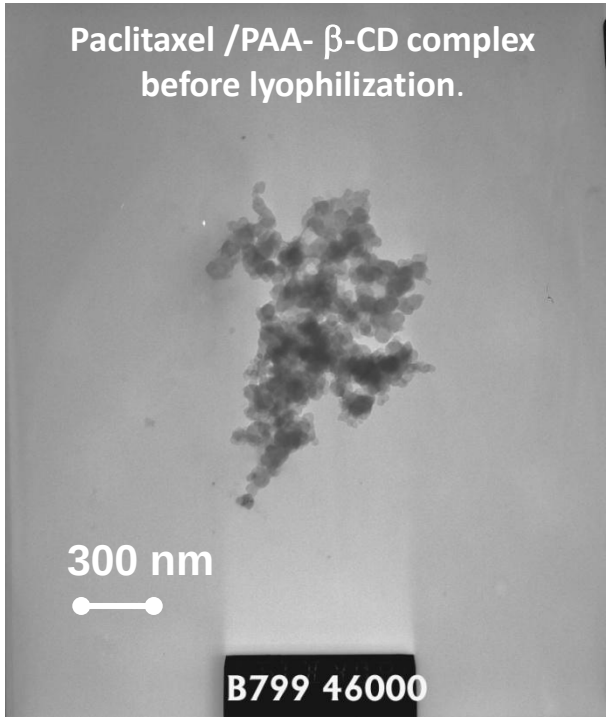


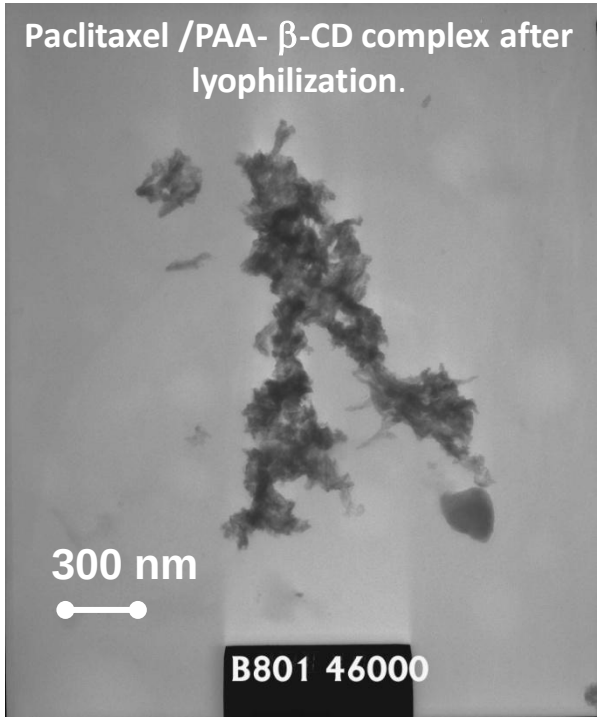
Preliminary biological evaluations carried out for hyperbranched PAA-β-CD including In-vitro MCF-7 cell viability tests and In-vivo haemolytic activity (human RBC), have confirmed the biocompatibility of the polymer.

PAA-β-CD/PACLITAXEL NP



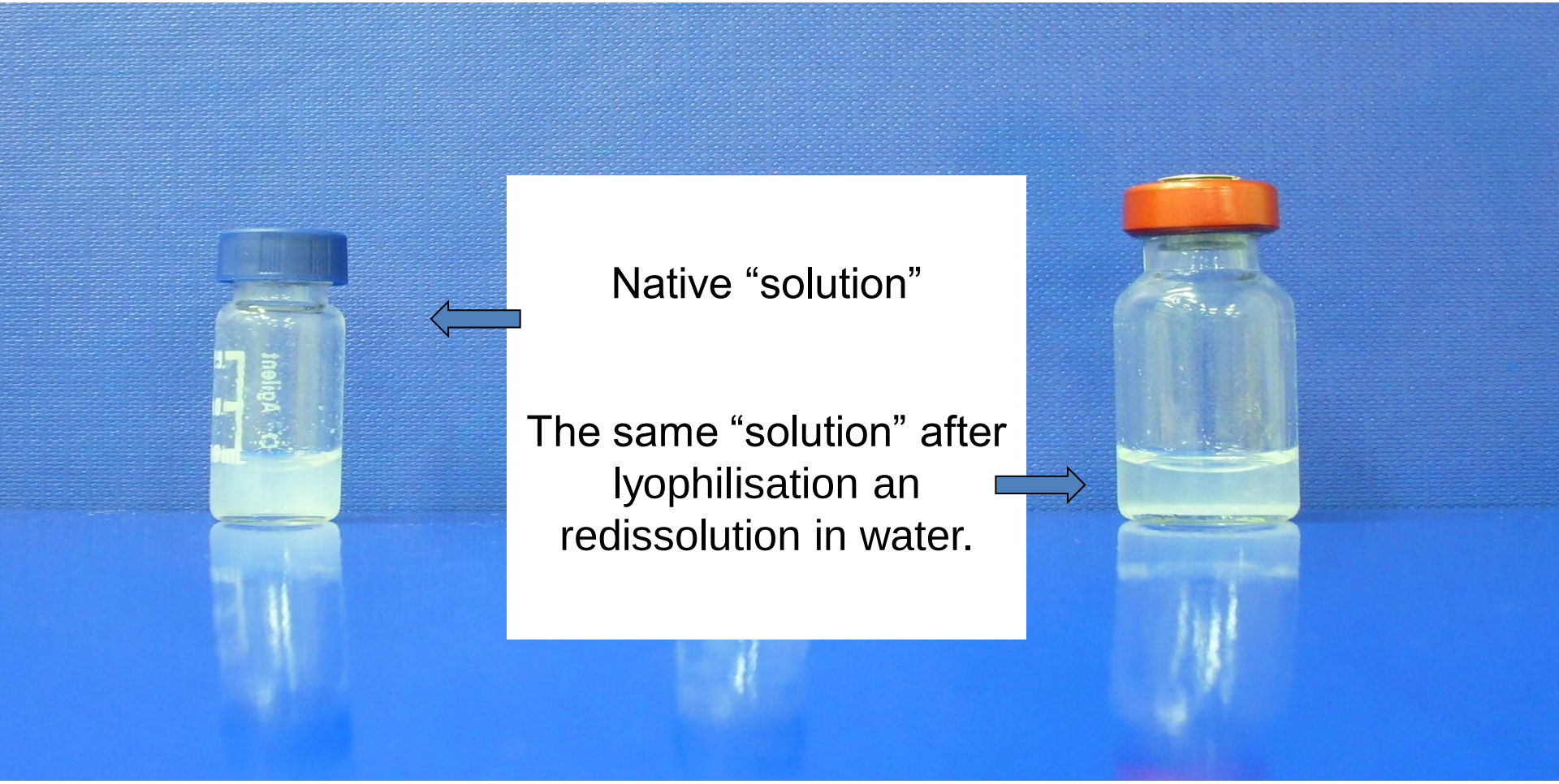
PACLITAXEL



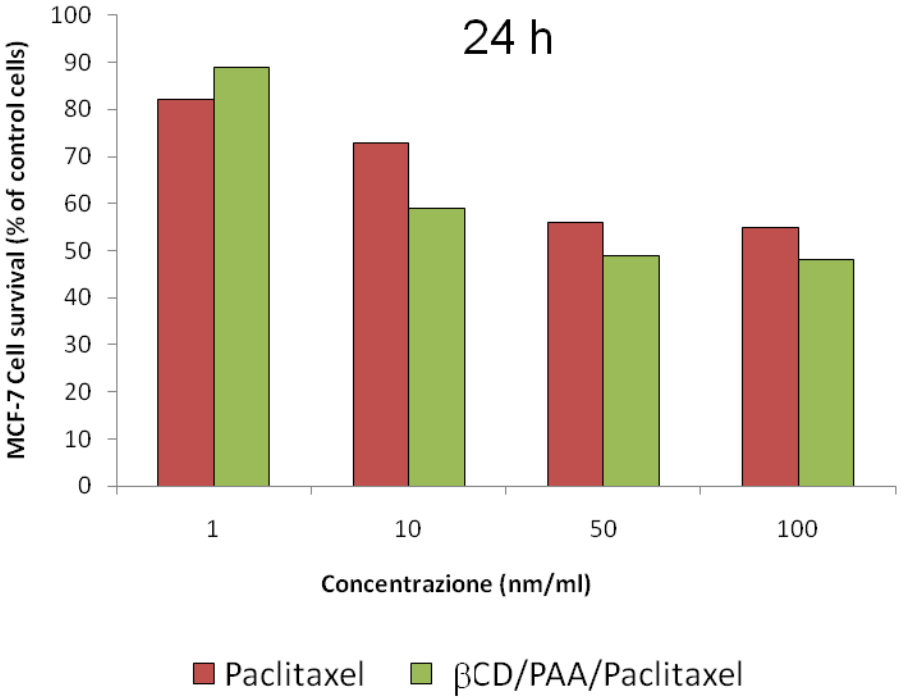


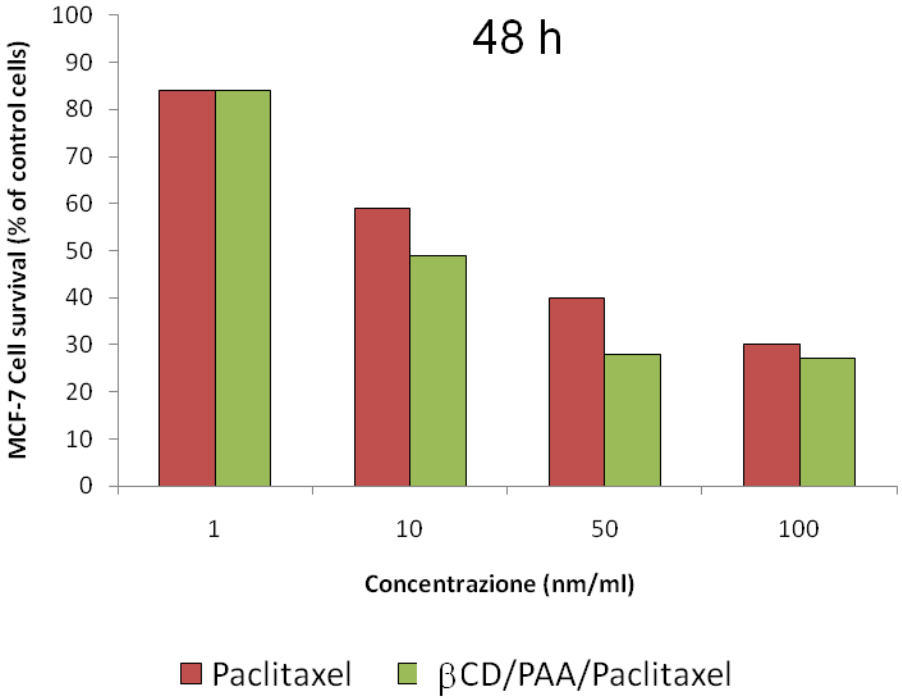
PAA-β-CD can be loaded with Paclitaxel up to 5% of their own weight forming nanoparticles with diameter < 500 nm

PAA-β-CD/PACLITAXEL NP



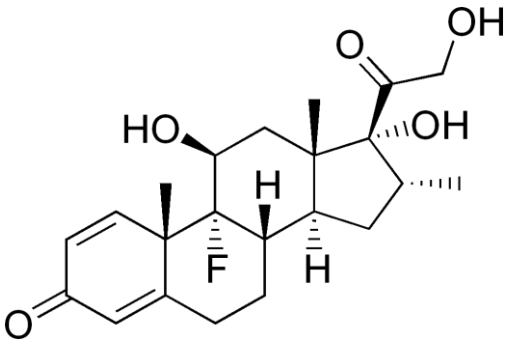
PAA-β-CD/PACLITAXEL NP: TOXICITY



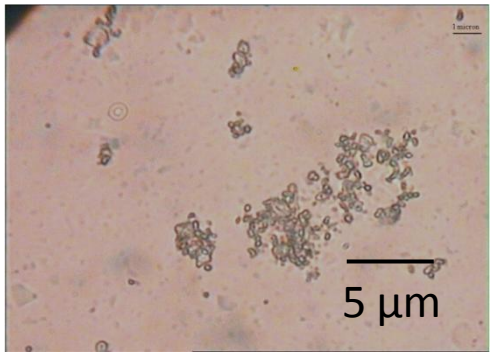


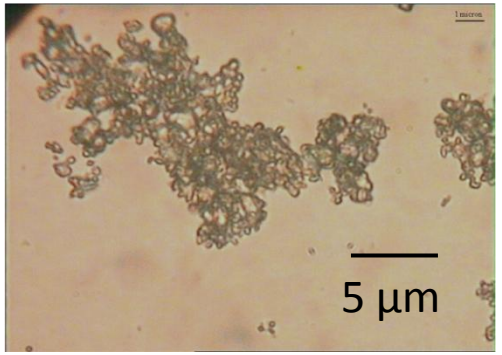
Paclitaxel even complexed by the polymeric system, mainteins its efficiency in the inhibition of cancer cell growth (In vitro MCF-7 cell cultures).

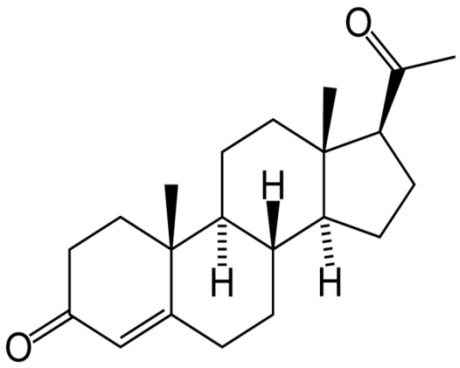
PAA-β-CD NANOPARTICLES



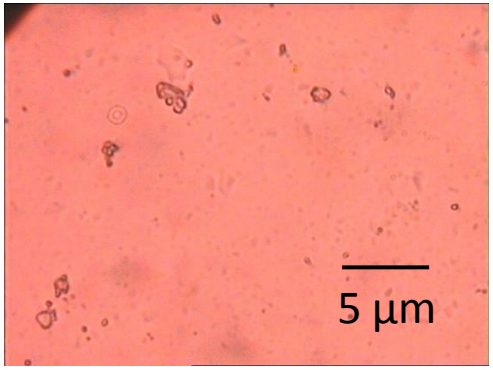
DEXAMETHASONE

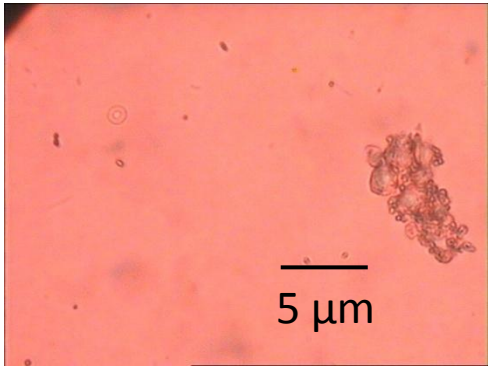






PROGESTERONE





Biomedical and Related Applications of Second Generation Polyamidoamines

1. Synthesis and properties of PAAs
2. PAAs as cytoplasmic delivery vehicles of immunotoxines
3. PAA-cholesterol nanoparticles
4. PAA-β-Cyclodextrin nanoparticles
5. **Conclusions**

CONCLUSIONS

PAAs constitute a very versatile family of ionic polymers that are easily synthesized and can be designed to be biocompatible and degradable in the body fluids.

They warrant potential, inter alia, as intracytoplasmic delivery vehicles of protein, as nanoparticoles for the release of lipophilic durgs and many other applications in the nanomedicine filed.

As a final observation, the unique combination of biotechnologically relevant properties of PAAs are still waiting to be fully exploited.

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	Agnese Bisazza

Thank You

for your

Kind Attention