Biodegradable Starburst Carbon Chain Polymer-Protein and Lysine Dendrite Conjugates: Adjustment of Immune Properties, DNA Bonding and Delivery

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Summary: In the past decade, the development of gene therapy technology has focused on the design of new nonviral carriers for gene delivery. Proteins modified with polyethyleneimine[1] or polylysine[2] as well as dendrites[3] have shown to be perspective carriers for DNA targeted delivery. The usage of protein conjugates as carriers of biologically active compounds will depend on the adjustment of their immune properties. To investigate this we have prepared starburst carbon chain polymer/protein conjugates containing low molecular weight biologically active compounds, salsolinol and bradykinin, in the polymer moieties and studied their immune properties. We have shown that chemical structure of the polymer moiety determines the conjugate biodegradation as well as their immune properties. The starburst poly(N-vinylimidazole) transferring poly(N-vinylimidazole) and polylysine 3G lysine dendrite conjugates have been prepared. The study of their ability to bind DNA and to guarantee its targeted delivery have shown that they are perspective DNA carriers.

Keywords: amino acid dendrites; biodegradation; DNA bonding and targeted delivery; immune properties; proteins; starburst carbon chain polymer conjugates

Introduction

Proteins as carriers of biologically active compounds have some specifics since they possess intrinsic biological activity and can guarantee the targeted delivery of biologically active compounds connected to them to the required areas. However proteins are capable to activate the immune system of the organism. This is an undesirable phenomenon if the proteins are used as a vehicle that provides targeted delivery of biologically active compounds. Therefore, in this case the activation of the immune system must be prevented. In contrast, proteins may also be used as a part of semi-synthetic immunogen to enhance immune response against low molecular weight

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compounds connected with them. In this case, the activation of immune system is desirable. However, in addition to the enhancement of the immune response against low molecular weight hapten, formation of a pool of antibodies against the protein carrier is also observed. A titer of antiprotein antibodies considerably exceeds that of the antihapten antibodies[4].

Therefore the important issues related to the application of protein conjugates as biodegradable carriers of biologically active compounds is adjustment of their immune properties. To solve this problem we synthesized and studied starburst carbon chain polymer protein conjugates.

Synthesis of Starburst Carbon Chain Polymer/Protein Conjugates (SCPPCs)

Increasing interest is being devoted to the preparation of SCPPCs in which the modifying carbon chain polymer is bound to a modified protein only at a single point [5-9]. The synthesis of SCPPCs includes typically two steps [6-9]. First, the carbon chain oligomers containing one carboxyl group are prepared either using a carboxyl group containing free radical initiator or employing a chain transfer approach with β-mercaptopropionic acid/AIBN initiating system. In the second step, the carboxyl groups of the carbon chain oligomers are activated and SCPPC containing modifying carbon chain olygomers attached by its carboxyl terminal group to a modified protein in a single point can be prepared.

Previously we developed the general method of the synthesis of SCPPCs with single-point attachment of modifying carbon chain polymer to modified protein[10-15]. The method consists of the synthesis of a macroinitiator based on proteins by their modification with dimethylimidate, or diazide 2,2'-azo-bisisobutyric acid (Fig.1, path A). In the second step, the protein macroinitiator is used in the polymerization of vinyl monomers for the formation of SCPPC, in which the modifying polymer is attached to the modified protein by one of its ends at a single point (Fig.1, path B). We used this approach with some alterations for obtaining polymeric conjugates of synthetic peptides[16] and biodegradable A-B and A-B-A block-copolymers where A is the polypeptide and B is the carbon chain polymer block[17,18]. Using this approach we obtained SCPPCs of insulin[10,12,14], trypsin[11,13] and horse-radish peroxidase[15] where poly(N-

vinylimidazole), poly(N-vinylpyrrolidone), polyacrylic acid and poly(acrylamide) and copolymer of N-vinylpyrrolidone with acrolein diethyl acetal were used as modifying polymers.

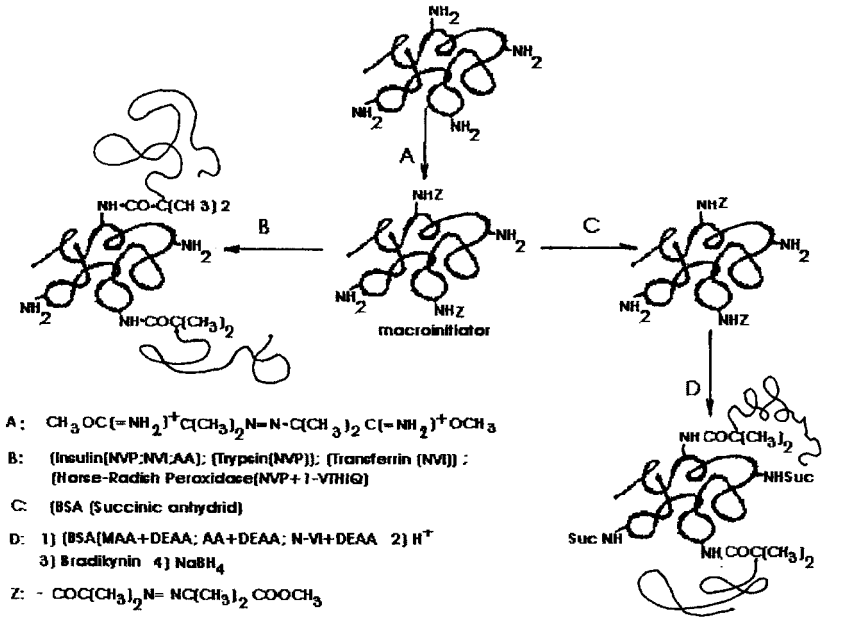


Figure 1. Scheme for the synthesis of starburst carbon chain polymer protein conjugates.

Biological Activity and Immunoreactivity of SCPPCs

The advantage of SCPPCs is the possibility of control of the number and the molecular weight of the polymer chains grafted to the protein as well as the position of modification for low molecular weight proteins, for example, for insulin[10,12,14]. We have found the possibility of decreasing the hormone immunoreactivity while preserving a considerable fraction of its hormonal activity. The data of Table 1 shows that PVI-insulin conjugates are generally more active. However, the chemical nature of the modifying polymer influences the biological activity of the conjugates up to 4 000 – 5 000 Da. At higher molecular masses of polymer modifier only the molecular weight and the position of the modified amino acid of the hormone influence its activity.

Table 1. Biological properties of starburst carbon chain polymer-insulin derivatives [10].

Starburst carbon chain polymer insulin conjugate	Modifier Mw x 10^{-3}	Biological activity	
		Decrease of glucose level in rabbits blood, %	Immunoreactivity, %
Insulin	-	100	100
A1, B29-AIB*	0.2	100	44
A1, B29-PVI	1.5	150	1.5
A1, B29-PVI	14	45	0.5
A1, B29-PVP	3	70	2.0
A1, B29-PVP	6	50	0
A1, B29-PAA	4	80	2.5
A1, B29-PAA	10	48	0.2
A1, B29-PA	2.5	56	3.5
A1, B29-PA	12	20	0
B1-PVI	1	95	5
B1-PVI	8	70	1.3
B1-PAA	5	65	3.0
B29-PVI	2	-	3.2
B29-PVI	6	-	2.0
B29-PAA	10	-	1.2
A1, B1, B29-PVI	4	0	0
A1, B1, B29-PAA	11	40	2.4

*Two point modified, A1,B29 2,2'-azo-bisisobutyryl intramolecular crosslinked insulin; PVI – poly(N-vinylimidazole); PAA –poly(acrylic acid); PA – poly(acrylamide)

Enzymatic Degradation of Starburst Carbon Chain Polymer Protein Conjugates

In *vitro* enzyme hydrolysis experiments with A1, B29-polymer insulin conjugates (Table 2) indicate that the control of the clearance time using its degradation by a model protease, trypsin, depends on chemical nature of the modifying polymer[10]. The choice of A1, B29-polymer/insulin conjugate and the usage of high specific enzyme allowed for studying the kinetics of the peptide bond hydrolysis in position B22-B23 (Arg-Gly) of the insulin derivatives and to determine the effect of the chemical nature and molecular weight of the polymer modifier on the formation of enzyme-substrate complex (K_m(app)) and the maximum hydrolysis rate, V_{max}. It was found that maximum hydrolysis rate, V_{max}, for all the insulin derivatives is practically independent of both the chemical nature of the modifying polymer and its molecular weight. The

data of Table 2 show that a great difference exists in K_m(app) for conjugates depending on the chemical nature of the polymer modifier. In this respect, PAA and PVP derivatives differ from PVI derivatives by three orders of magnitude.

Table 2. Kinetic data for trypsin hydrolysis of A1, B29-starburst carbon chain polymer/insulin conjugates.

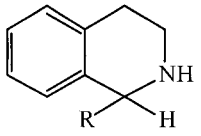
Insulin derivative	Modifier Mw x 10^{-3}	K_m(app) x 10^6	V_{max} x 10^{10} mol min^{-1} mg^{-2}
Insulin	-	4 500	1.9
A1, B29- AIB	0.3	2 200	3.1
A1, B29- PVI	6	3 500	3.2
A1, B29- PVI	10	2 000	1.4
A1, B29- PVI	14	6 300	4.1
A1, B29- PAA	4	1.8	1.8
A1, B29- PAA	6	6.6	2.5
A1, B29- PAA	8	5.4	0.8
A1, B29- PVP	6	1.3	2.8
A1, B29- PVP	22	3.8	1.0

A sharp difference in K_m(app) between PAA and PVI derivatives may be caused by several reasons. One of them is the difference between the total charge of polymer-insulin conjugates: it is negative for PAA and positive for PVI derivatives. This facilitates the formation of the enzyme-substrate complex in the former case and makes it difficult in the latter case because of the positive charge of trypsin: its isoelectric point is 9.6[19]. PVP can also favour the formation of the Michaelis-Menten complex since the PVP ability for forming complexes with proteins is known. The data of Fig.2 also show that in the considered range the change in the molecular weight of the modifying polymer can be seen up to molecular weight 5 000 – 6 000 Da and then virtually does not affect K_m(app).

Immune Properties of Starburst Carbon Chain Polymer Protein Conjugates[20]

To investigate the possibility of adjusting the immune properties of SCPPCs, we have prepared SCPPCs containing model low molecular weight biologically active compounds, salsolinol (THIQ) and peptide hormone bradykinin (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) in the polymer

moiety. We have developed two different approaches for their preparation. The first approach (polymerization approach, Fig.1, path B) included preparation of SCPPC's by co-polymerization of N-vinylpyrrolidone and 1-vinylsalsolinol (VTHIQ) with usage of a macroinitiator based on horse-radish peroxidase (HRP) previously modified with 2,2'-azo-bisisobutyric acid (Fig.1, paths A, B).



R = CH_3 methyl-1,2,3,4-tetrahydroisoquinoline, Salsolinol

R = $CH{=}CH_2$ 1-vinyl-1,2,3,4-tetrahydroisoquinoline, 1-Vinylsalsolinol

In an attempt to synthesize SCPPC's based on bovine serum albumin (BSA) containing bradykinin in the polymer moiety of the conjugate by co-polymerization of N^{α}-methacrylyl-bradykinin ($CH_2{=}C(CH_3)$-CO-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) with N-vinylpyrrolidone, a conjugate was obtained that did not contain bradykinin. This failure can be explained by the considerable decrease of vinyl bond reactivity in the N^{α}-methacrylyl-bradykinin. To bypass this failure, we used the polycondensation approach. It involves the preparation of SCPPCs based on BSA containing co-polymers of acrolein diethyl acetal with methacrylic acid, N-vinylpyrrolidone and N-2-methyl-vinylimidazole in the polymer moiety of the conjugate (Fig. 1, paths A, C and D). The deprotection of reactive aldehyde groups in acrolein diethyl acetal fragments of the polymer moiety is achieved simultaneously during the chromatographic purification of the conjugates using buffer at pH 3.5. Conjugates containing reactive aldehyde groups of acrolein repeat units in the carbon chain polymer allowed to prepare SCPPCs containing bradykinin. The introduction of additional succinic groups of macroinitiator preparation were a specific feature of bradykinin containing SCPPCs. Their presence should probably increase immunogenicity of SCPPCs [21-23]. These groups appeared after additional modification of residual amino groups of proteins which remained free after their interaction with fragments of derivatives of 2,2'-azo-bisisobutyric acid at the stage of macroinitiator preparation (Fig. 1, path A, C).

Immune Properties of Starburst Carbon Chain Polymer Protein Conjugates

Titers observed after immunization of rabbits by salsolinol-containing SCPPCs are presented in Table 3. Salsolinol containing BSA conjugates prepared from succinylated BSA and THIQ with the help of water soluble carbodiimide were used as control conjugates. It is obvious that anti-salsolinol antibody titers originated from immunization with starburst conjugates were considerably higher. Moreover control antisera were enriched mainly with antibodies against protein carrier (BSA), rather than against the hapten (salsolinol).

Table 3. Antibody's titer against THIQ, VTHIQ and BSA [20].

Immunogen	Hapten, mol.%	Antigen	Titer (ELISA)
$THIQ_{10}$-BSA(Suc)*	THIQ, 10	BSA	204800
		$THIQ_{13}$-Ova**	ABs are absent
$THIQ_{20}$-BSA(Suc)*	THIQ, 20	BSA	192400
		$THIQ_{13}$-Ova**	160
Poly(VP_p,$VTHIQ_q$) –HRP	VTHIQ, 5	$THIQ_{13}$-Ova**	640
Poly(VP_p,$VTHIQ_q$) –HRP	VTHIQ, 3	$THIQ_{13}$-Ova**	2560

* Prepared from succinylated BSA and THIQ with the help of water soluble carbodiimide.
** Prepared from Ova and THIQ with the help of glutaraldehyd.

The chemical structure of the polymer moiety of conjugates does determine the immune properties of the conjugates. It follows (Table 4) that SCPPC based on BSA containing bradykinin in the polymethacrylic acid moiety of the conjugate gave maximal antibodies titer against bradykinin and BSA, as compared to control data for bradykinin containing succinylated BSA ('standard" conjugate). Enhanced immune response against the components of SCPPCs containing polymethacrylic acid may be caused by the ability of polyanionic polymers to activate immune system[21-23]. SCPPC containing poly(N-vinylpyrrolidone) as polymer modifier gave also rather high titer of antibradykinin of antibodies but diminished titer antibodies against BSA. Interestingly poly-N-2-methyl-vinylimidazole as a polymer moiety of SCPPC decreases the

formation of antibodies against bradykinin as well as against the protein carrier. Reduction of immunogenicity of SCPPC, which has poly-N-2-methyl-vinylimidazole fragments bound to succinylated BSA, was probably caused by significant neutralization of its total charge.

DNA Bonding and Targeted Delivery [24]

Taking into account the possibility of diminishing the immune response against polymer-protein conjugates by the use of poly(N-vinylimidazole) as modifying polymer we have prepared series of SCPPCs based on transferring and lysine- dendrite of the third generation (3 G lysine dendrite), namely poly(N-vinylimidazole) transferring ($p(VI)_nTf$), poly(N-vinylimidazole) 3G lysine dendrite ($p(VI)_nD1$) and polylysine 3G lysine dendrite ($p(Lys)_nD1$) according to scheme of Fig. 1 (path A and B).

Table 4. Antibody's titer against bradykinin and BSA[20].

Immunogen	Antigen			
	Immunization with Freund's adjuvant		Immunization without Freund's adjuvant	
	BK-Ova***	**BSA**	BK-Ova ***	**BSA**
Poly(MAA_k, $DEAA_l$ BK_h)-BSA(Suc)	5120	102400	320	2560
Poly(VP_k, $DEAA_l$ BK_h)-BSA(Suc)	2560	3200	*	160
Poly(VMI_k, $DEAA_l$ BK_h)-BSA(Suc)	40	640	**	**
BK_h - BSA(Suc)	2560	6400	320	2560

* Titer was not determined; ** Immunization was not made; *** Prepared from ovalbumin and bradykinin with the help of glutaraldehydl

3G Lysine dendrite (D1) and 3G lysine dendrite containing palmitoyl fragments on its C-terminal (D2) were prepared with the usage of solid phase peptide chemistry approach (benzhydrylamino-polymer, BOC/TFA strategy). Poly(lysine) derivative of 3-G lysine dendrite ($p(Lys)_nD1$) was prepared by the polymerization of Leuchs' anhydride of N^{ε}-carbobenzyloxycarbonyl-lysine on the amino groups of 3G-lysine dendrite[25] as initiating groups, followed by the removing of carbobenzyloxy groups.

Gel Retardation Assay[24]

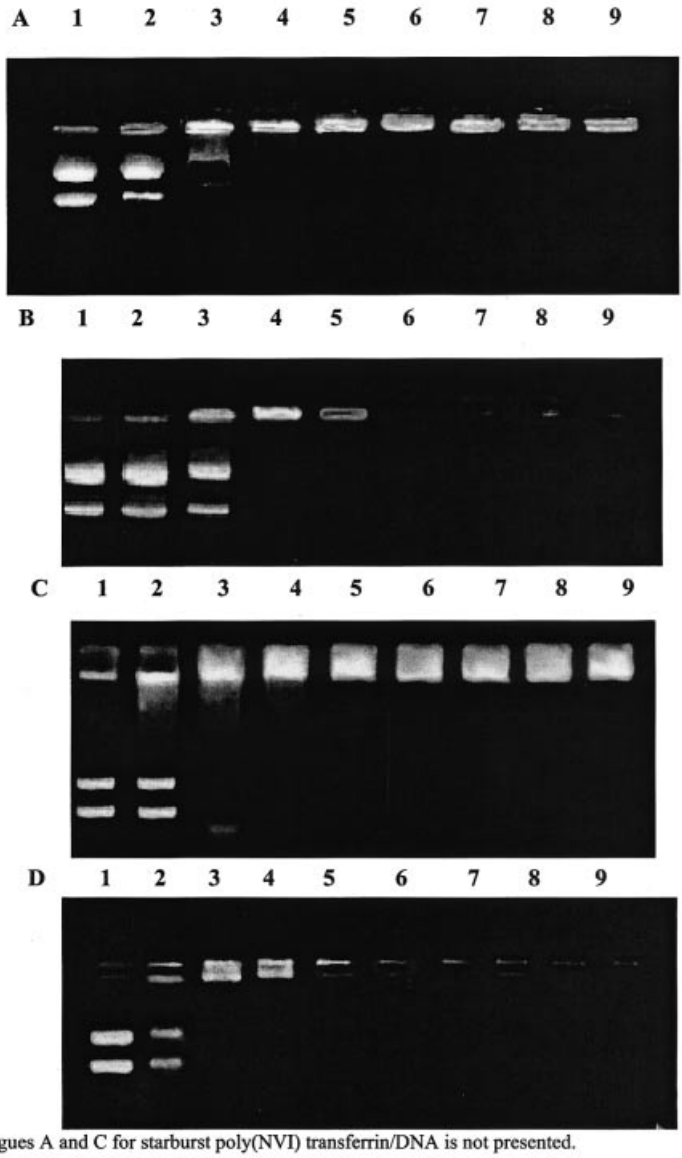
The gel retardation assay shows the ability of starburst poly(N-vinylimidazole) transferring, poly(N-vinylimidazole) and polylysine 3G lysine dendrimers to form complexes with DNA (pCMV-nlsLacZ) (Tab. 5 and Fig. 2). The ability of forming stable complex of transferrin (Tf) conjugates depends on the molecular weight and amount of grafted polymer chains (Table 5). All SCPPCs based on lysine dendrimer form complexes with DNA under the relationship carrier/plasmid DNA (pCMV-nlsLacZ) 1: 1. However there are some peculiarities. Binding abilities of carriers based on D1 to form complexes with plasmid DNA were monitored by fluorescent titration assay with ethidium bromide.

Table 5. Gel retardation data for DNA*)/carrier complexes.

Structure	Carrier Mw	DNA/ carrier
$(Lys)_8$-$[Lys]_4$-$(Lys)_2$-LysAla-NH_2 (D1)	-	1:1
$(Lys)_8$-$[Lys]_4$-$(Lys)_2$-Lys –(Lys(N^{ε}-Pal) $_2$- Ala-NH_2 (D2)	-	1:1
$[p(N\text{-}VI)_n]_m$-$(Lys)_8$-$[Lys]_4$-$(Lys)_2$-Lys-(Lys(N^{ε}-Pal)$_2$-Ala-NH_2 ($p(VI)_n$ -D1)	80 000	1:1
$[p(Lys)_m]_n$- $(Lys)_8$-$[Lys]_4$-$(Lys)_2$-Lys-AlaNH$_2$ ($p(Lys)_n$-D1)	22 000	1:1
p(N-VI)$_6$- Tf	18 000	1:500
p(N-VI)$_{15}$- Tf	57 000	1:250
p(N-VI)$_{16}$- Tf	84 800	1:50
p(N-VI)$_{16}$- Tf	96 000	1:10
p(N-VI)$_{17}$ - Tf	34 000	1:50
p(N-VI)$_{19}$ - Tf	38 000	**

* plasmid DNA : pCMV- nlsLacZ; **Complex was not formed

For the D2 (3G lysine dendrimer containing lipophilic fragments on the C-termial fragment) and starburst poly(lysine) conjugate of 3G lysine dendrimer D1, the fluorescence of the intercalated dye was absent at 2:1 carrier/plasmid DNA ratio (pCMV-nlsLacZ) (Fig. 2, B and D). Evidently in these cases very compact complexes were formed excluding intercalation of the dye in plasmid DNA. For D1 (Fig. 4, A) and for $p(VI)_n$D1 (Fig. 4, C) intercalation of ethidium bromide was constant, independent of the carrier/plasmid DNA (pCMV-nlsLacZ) ratio.



* Data analogues A and C for starburst poly(NVI) transferrin/DNA is not presented.

Figure 2. Stability of dendrimer /plasmid DNA (pCMV-nlsLacZ) complexes for D1 (A); D2 (B); p(N-VI)$_n$-D1 (C) and p(Lys)$_n$D1 (D)*: 1-"naked"DNA; 2- 1/0.1; 3- 1/0.5; 4- 1/ 1; 5- 1/2 ; 6- 1/4; 7- 1/6; 8- 1/8; 9- 1/10.

Transfection Properties [24]

Preliminary data on the possibility of transfection with the usage of starburst poly(NVI) dendrite D1 conjugate have shown that 3 G lysine dendrimer (D1) and its C-terminal derivate (D2) containing lipophilic fragments and N-terminal derivate – starburst poly(N-vinylimidazole) conjugate (poly(NVI)$_n$-D1 do guarantee targeted delivery to DNA in cells. After 24 hr from the transfection of epitalial carcinoma HeLa with DNA/carrier, DNA was observed in cells. (Fig. 3, A, B, C).

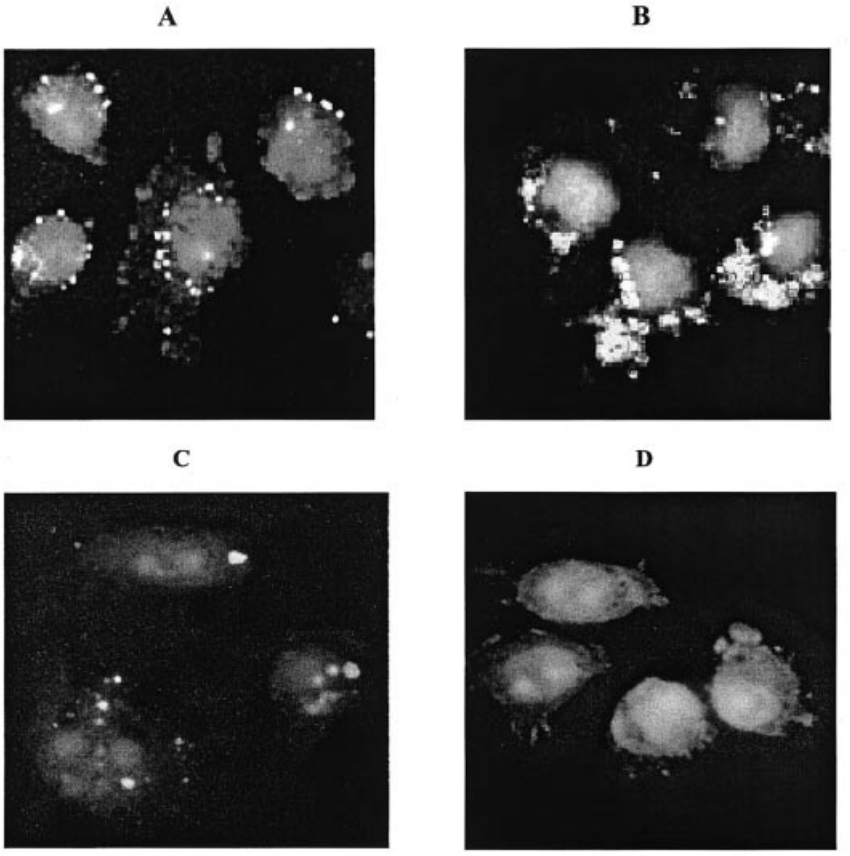


Figure 3. Localization of DNA/carrier complexes (D1 (A); D2 (B); p(N-VI)$_n$ -D1(C)) 24 hrs after transfection cells of epitalial carcinoma HeLa. (D) – intact, without complex transfection cells.

Conclusion

Synthesis of biodegradable starburst carbon chain polymer/protein and lysine dendrimer conjugates was carried out. It was found that the polymer moiety of the conjugates determines both their biodegradability and their immune properties. Variation in the chemical structure of modifying carbon chain polymer allows for enhancing or suppressing the immunogenicity of starburst carbon chain polymer conjugates based on proteins. This may constitute a useful tool in preparation of semi-synthetic immunogenes for the production of high level of specific antihapten antibodies for immunological analysis or creation of artificial vaccines. This may also provide new possibilities in the production of new pharmaceuticals with suppressed immunological inactivation of medicals. It was shown the ability of starburst carbon chain polymer conjugates based on proteins and lysine dendrites to form complex with plasid DNA and to guarantee its targeted delivery into cells.

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