(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
24 June 2021 (24.06.2021)

WIPO | PCT

(10) International Publication Number
WO 2021/121446 A1

(51) International Patent Classification:
A61K 47/58 (2017.01)

(21) International Application Number:
PCT/CZ2020/050097

(22) International Filing Date:
16 December 2020 (16.12.2020)

(25) Filing Language: Czech

(26) Publication Language: English

(30) Priority Data:
PV 2019-778 17 December 2019 (17.12.2019) CZ

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, IT, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

WO 2021/121446 A1

(54) **Title:** COPOLYMER WITH HYDROLYTIC RELEASE OF CANCEROSTATIC AGENT CYTARABINE, METHOD OF ITS PREPARATION AND USE THEREOF

(57) **Abstract:** The present invention relates to the preparation, structure and properties of water-soluble polymer therapeutics based on cytarabine derivatives, intended primarily for the treatment of leukaemias and non-Hodgkin's lymphomas in human medicine. Cytarabine is attached to the polymer backbone via amide bond to an amino acid or oligopeptidic sequence situated within the polymer side chain. The structure of cytarabine acylamides enables the stability of the whole polymeric system during blood circulation, and thus its sufficient accumulation in the neoplastic tissue, wherein the drug is allowed to be released hydrolytically. The present invention further relates to the polymeric cytostatics, which is stable in blood stream, to the pharmaceutical composition and use thereof in medicine.

Copolymer with hydrolytic release of cancerostatic agent cytarabine, method of its preparation and use thereof

Field of Art

The invention relates to the structure and properties of water-soluble polymer therapeutics based on cytarabine derivatives intended primarily for the treatment of leukaemias and non-Hodgkin's lymphomas in human medicine.

Background Art

Recently, drug development trends have very often focused on the development of dosage forms that allow for a specific drug effect only at the site of the desired therapeutic effect. Such targeted biologically active substances find application especially in areas where undesirable side effects of the drug can lead to damage to healthy parts of the organism and/or where the drug itself is unstable in the organism and its degradation is a limiting factor of its effectiveness. These dangers are particularly acute in the treatment with cytotoxic agents in cancer chemotherapy. It is known that the attachment of a cytostatic to a water-soluble polymeric carrier by chemical bonding makes it possible to increase the solubility of otherwise insoluble or poorly soluble drugs, significantly reduce their direct toxicity and prolong circulation time in the bloodstream, both by increasing the stability of carried molecules against degradation and due to increased molecular weight of the carrier. The high molecular weight of the polymers prevents the rapid elimination of the drug from the body by glomerular filtration, and thus ensures a prolonged circulation time in the blood and residence in the organism, and thus a longer bioavailability of the drug.

In the past, many polymeric conjugates of anticancer drugs with soluble polymers have been prepared and studied, in which a drug with antitumor effect has been attached to the polymer by a non-cleavable covalent bond, a hydrolytically unstable ionic bond or a covalent bond enabling controlled release of the drug and thus its activation by enzymatic or simple chemical hydrolysis of this bond. In general, polymeric carrier systems are designed to be capable of releasing a therapeutically active anticancer drug from a carrier either in a tumour, or more specifically, directly in a tumour cell. An important group of polymer therapeutics are polymeric drugs prepared on the basis of copolymers of *N*-(2-

hydroxypropyl)methacrylamide (HPMA), many of which are actively targeted to tumours through a targeting structure (antibody, lectin, hormone) attached to the polymer. In the development of polymer cancerostatics, it has also been demonstrated that it is not necessary to use an actively targeted carrier for specific drug delivery to the tumour site, but significantly increased accumulation of polymer cytostatics, especially in solid tumours, can be achieved by increasing the molecular weight of the polymer therapeutic (so-called passive targeting to solid tumours). This ability of macromolecules to accumulate in solid tumours has been termed the Enhanced Permeability and Retention (EPR) effect, and this effect has been shown to be also significant in HPMA copolymer-based carriers.

One of the main problems with the use of HPMA copolymers as passively targeted high-molecular-weight carriers is their non-cleavable carbon chain. Only polymers not exceeding a molecular weight of 40,000 to 50,000 g/mol can be eliminated from the body. That is, if the polymer is not to accumulate in the body after repeated administration of the drug, and if the molecular weight of the carrier is to be as high as possible to make passive targeting as effective as possible, the polymeric carrier must be designed to be degradable in the body. Such polymeric carriers based on HPMA copolymers have recently been developed and their structure has been patented [Chytil P, Etrych T, Koňák Č, Šírová M, Mrkvan T, Bouček J, Říhová B, Ulbrich K, New HPMA copolymer-based drug carriers with covalently bound hydrophobic substituents for solid tumour targeting, Journal of Controlled Release 127, 121-30 (2008), Etrych T, Strohalm J, Chytil P, Říhová B, Ulbrich K, Novel star HPMA-based polymeric conjugates for passive targeting to solid tumours, Journal of Drug Targeting 19(10), 874-89 (2011), CZ 302830 B6, CZ 298 945 B6].

There is a wealth of information in the literature on the preparation and study of the properties of polymers bearing a cancerostatic attached to the polymer by a bond susceptible to hydrolysis in an aqueous medium. Among them, HPMA copolymers carrying the cancerostatic drug doxorubicin bound to the polymer chain by a hydrolytically cleavable hydrazone bond play an important role [Etrych T, Chytil P, Jelínková M, Říhová B, Ulbrich K, Synthesis of HPMA Copolymers Containing Doxorubicin Bound via a Hydrazone Linkage. Effect of Spacer on Drug Release and in vitro Cytotoxicity. Macromolecular Biosci. 2, 43-52 (2002), CZ 293787 B6]. This bond is relatively stable in the bloodstream environment (during transport in the body) and hydrolytically labile in the slightly acidic environment of a living cell. The rate of hydrolysis of this bond also controls the rate of drug release, and thus the concentration of active substance at the site of desired effect.

In the treatment of haematological malignancies, cytarabine (araC) is often used as a drug for nucleoside analogues as antimetabolites and one of the longest and most widely used. Cytarabine is one of the main anti-leukaemic cytostatics and has an irreplaceable role in the treatment of non-Hodgkin's lymphomas.

It is used both in the first-line treatment and as part of consolidation and rescue regimes. It is cytosine arabinoside, where cytosine is linked to an arabinose sugar. Because cytarabine is sufficiently structurally similar to deoxycytosine, it can be incorporated into DNA instead. However, cytarabine must first enter the tumour cell. At low plasma concentrations, cytarabine is predominantly actively transported into the cell by transporters Solute Carrier Family 29 Members 1 and 2 (SLC29A1, SLC29A2). Upon reaching high plasma concentrations (~ 10 μM), cytarabine gets into tumour cells passively according to a concentration gradient. Cytarabine is itself a prodrug without a direct antitumor effect. Inside tumour cells, cytarabine must first be activated by sequential phosphorylation to cytarabine triphosphate and then incorporated into DNA. A key step in activating cytarabine is thought to be its phosphorylation by the enzyme deoxycytidine kinase (dCK) to cytarabine monophosphate. Repeated studies have shown that the acquired resistance of tumour cells to cytarabine is due to a decrease in dCK expression. The metabolism, activation and deactivation of cytarabine and its metabolites is a highly complex process and depends, in addition to the dose and route of administration, on the microenvironment (different concentrations of cytidine deaminase in different parts of the cell) and on congenital genetic factors.

Cytarabine is one of the cytostatics that specifically affect the S phase of the cell cycle, as it strongly interferes with DNA synthesis and thus damages DNA in the replication phase. Due to their high rate of proliferation, rapidly dividing cells of haematological malignancies are far more affected than healthy cells. The main degradation mechanism of cytarabine and its metabolites is deamination, in particular by the enzyme cytidine deaminase to the corresponding inactive uracil derivatives. Due to the rapid degradation, cytarabine is therefore dosed either in repeated short-term infusions at high doses (so-called high-dose cytarabine, usually 2-3 grams of araC/m^2 of the patient's body surface area given in a short-term 2-3 hour infusion, 4 to 6 applications after 12 hours), or is administered continuously in significantly lower doses (usually 100 - 200 mg araC/m^2 in a continuous infusion usually for 7 days). Palliative therapy for acute leukaemias and myelodysplastic syndromes also uses repeated subcutaneous applications of small doses of cytarabine (usually 20 mg araC s.c. twice daily for 10 days). The pharmacokinetics of cytarabine therefore has a crucial impact on its anti-tumour

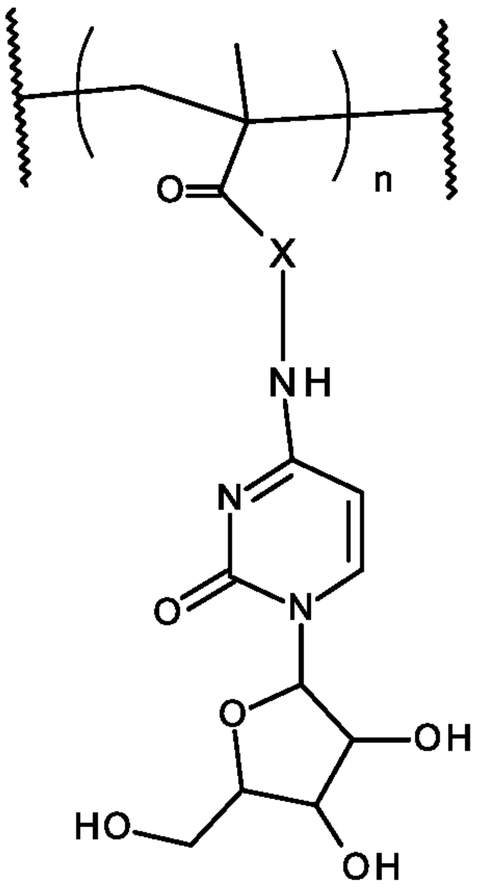
effect and toxicity (especially haematological). Intrathecal applications of small doses of cytarabine (50 mg) are used to treat patients with central nervous system disorders with meningeal leukaemia or lymphoma. In 1999, liposomal cytarabine (DepoCyt®) was approved in the United States for intrathecal administration in the indication of lymphomatous or leukaemic cells infiltrating the meninges to achieve longer exposure due to the gradual release of cytarabine from the liposome. Controlled clinical studies have shown that liposomal cytarabine is as effective as or more effective than cytarabine alone. In lymphoma meningitis, liposomal cytarabine offers an excellent response rate, improved quality of life of patients and prolonged time to neurological progression. If the cause of meningitis is a solid tumour, liposomal cytarabine will prolong the time to neurological progression and improve quality of life. However, Depocyt® was discontinued in 2017 due to unclear (unspecified technical) reasons, was withdrawn from the market and is therefore no longer available. Recently, a paper has been published describing the preparation of a system for the controlled delivery of cytarabine. An HPMA-based polymeric conjugate has been described in which cytarabine is linked via an oligopeptide linker in the side chains of a polymeric carrier [Pola R, Janoušková O, Etrych T, The pH-Dependent and Enzymatic Release of Cytarabine From Hydrophilic Polymer Conjugates, Physiol. Res. 65(2), 225-32 (2016)]. This polymeric conjugate demonstrated its cytotoxic activity reduced by one order of magnitude relative to free cytarabine in vitro on several lymphoma lines. The system hydrolytically released almost 50 % of the drug after 72 h of incubation in a pH 7.4 environment mimicking the bloodstream environment.

Summary of the Invention

The present invention relates to the structure, synthesis and use of a novel polymeric drug with a narrow molecular weight distribution ($Đ \approx 1.1$ to 1.4) and with a defined content of covalently bound cytarabine. The described polymeric drug is characterized by prolonged pharmacokinetics, increased accumulation in the tumour and significant anti-tumour activity. It is a random linear or star polymeric conjugate composed of monomeric units *N*-(2-hydroxypropyl) methacrylamide) and monomeric units carrying cytarabine attached to the polymer backbone via linkers that allow hydrolytic release of cytarabine in the human body.

An object of the present invention is a polymeric conjugate comprising from 80 to 95 mol% of monomeric units of HPMA and from 5 to 20 mol% of monomeric units of the general formula I and optionally II (based on the sum of monomeric units of HPMA, (I) and (II))

I



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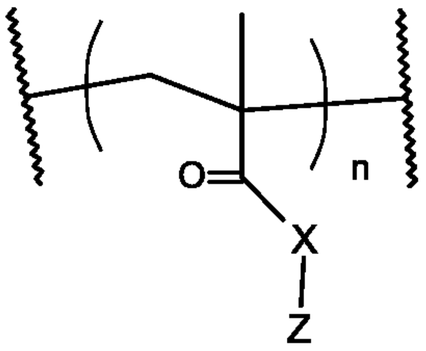
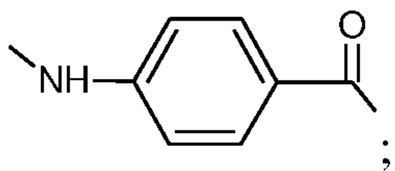


wherein

X is a covalent bond or an aminoacyl attached via an amide bond to the carbonyl group of the monomeric unit (I) and, optionally, to the carbonyl of the monomeric unit (II) and selected from the group consisting of

–NH–$(CH_2)_n$–C(=O)–, where *n* is an integer from 1 to 6; –NH–$(CH_2)_q$(C(=O)–NH–$(CH_2)_r)_p$–C(=O)–



, wherein *p, q* and *r* are independently selected from 1, 2 and 3;

;

–NH–$(CH_2)_n$–C(=O)–NH–N=C(A)–$(CH_2)_q$–L–C(=O)–; wherein *n* is an integer from 1 to 6; *q* is selected from 1, 2 and 3; A is selected from the group consisting of methyl, isopropyl, cyclohexyl and pyridyl; L is a covalent bond or 1,4-phenylene;

wherein –CH_2– of group X may be further substituted with one or more of the same or different natural amino acid side chains;

and/or the phenylene of group X may be further substituted with one or more of the same or different natural amino acid side chains;

preferably the chains of the natural amino acid are methyl, isopropyl, isobutyl, –$CH(CH_3)(CH_2CH_3)$, –CH_2OH, –$CH(OH)(CH_3)$, –CH_2–$(C_6H_4)OH$, –$(CH_2)_2$–S–CH_3,

–CH_2SH, –$(CH_2)_4$–NH_2, –CH_2COOH, –$CH_2C(O)NH_2$, –$(CH_2)_2COOH$,

–$(CH_2)_2C(O)NH_2$, –$(CH2)_3NH$–$C(=NH)(NH_2)$, benzyl;

with the proviso that X is not –NH–$(CH_2)_2$–C(=O)– and

X is not –NH–CH_2–C(=O)–NH–CH(CH_2–Ph)–C(=O)–NH–CH(CH_2–iPr)–C(=O)–NH–CH_2–C(=O)–

;

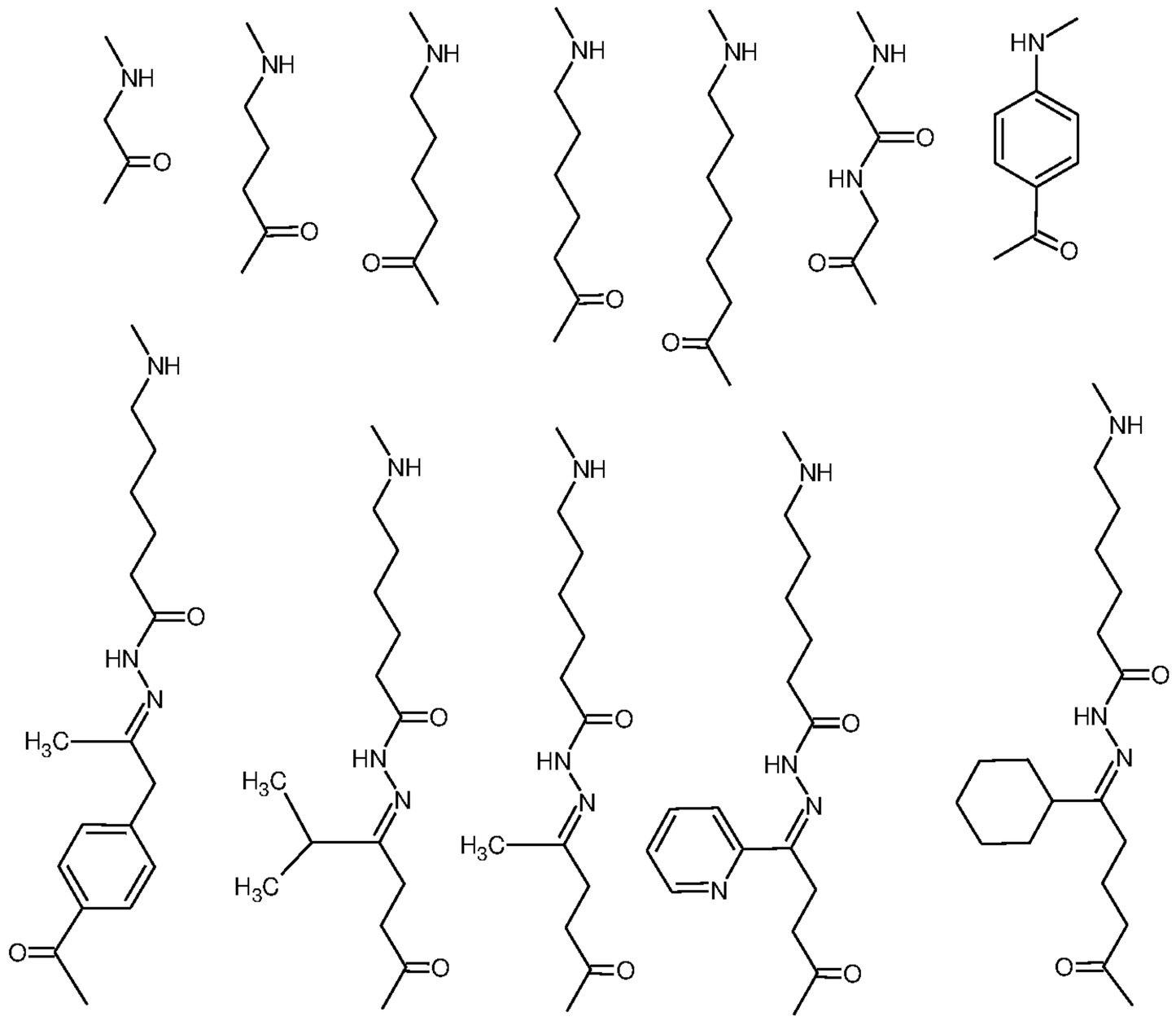
and Z is a reactive substitution derivative of X, preferably Z is selected from the group comprising –COOH; thiazoline-2-thion (TT); (4-nitrophenyl)oxy group; (2,3,4,5,6-pentafluorophenyl)oxy group; (succinimidyl)oxy group; hydrazide, –OH; -NH-CH_2-C(OH)-CH_3; –C(=O)–NH–$(CH_2)_a$–CH_2(OH); –C(=O)–NH–$(CH_2)_b$–CH(OH)–CH_3; –C(=O)–NH–$(CH_2)_b$–CH(OH)–$(CH_2)_c$–CH_3; wherein *a* is an integer from 0 to 4, *b* is an integer from 0 to 3 and *c* is an integer from 1 to 4;

wherein in total the monomeric units of the general formula (I) and of the general formula (II) are at most 20 mol%, and the total amount of the monomeric units of the general formula (I) is at least 5 mol%, based on the sum of monomeric units of the HPMA, (I) and (II);

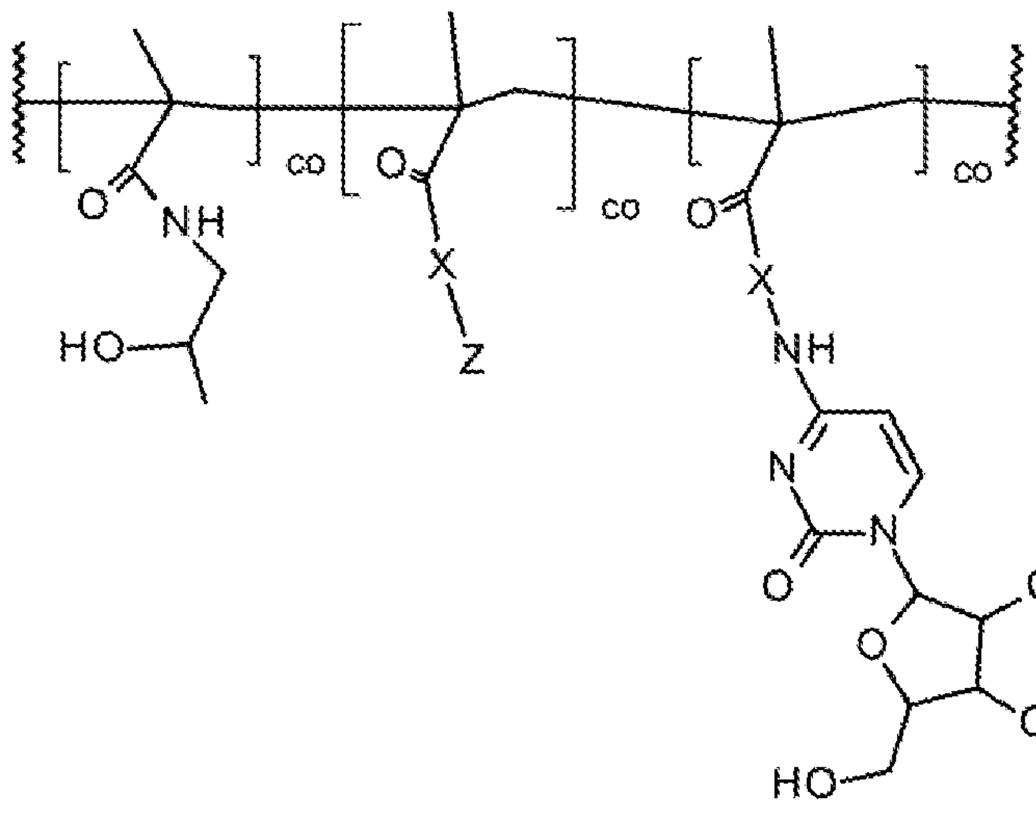
and wherein the molecular weight M_n of the polymeric conjugate is in the range of from 4,000 to 1,000,000 g/mol, preferably in the range of from 15,000 to 300,000 g/mol, more preferably the molecular weight is in the range of from 30,000 to 100,000 g/mol, most preferably from 40,000 to 80,000 g/mol.

By natural amino acids are meant histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine, arginine, cysteine, glutamine, glycine, proline, tyrosine, alanine, aspartic acid, asparagine, glutamic acid, serine, selenocysteine. The side chains are chains attached to the alpha-carbon of the amino acid.

In one preferred embodiment, X is a covalent bond or X is selected from the group consisting of:



In one embodiment, the polymeric conjugate is a linear statistical copolymer of HPMA and monomeric units of general formula (I) and optionally (II). This linear statistical copolymer can be represented by the general formula (III).



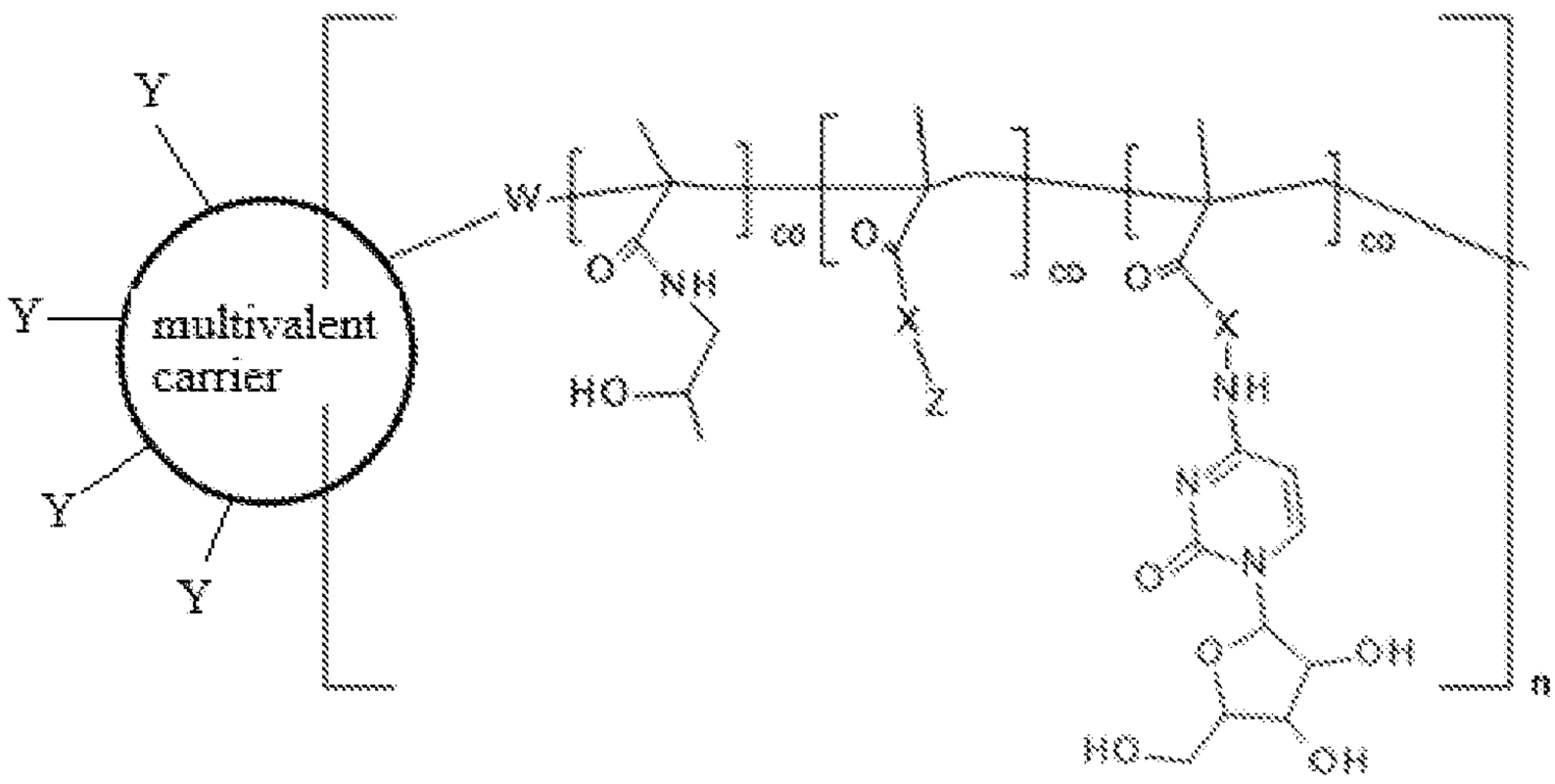
(III)

The molar mass of the linear statistical copolymer is from 4,000 to 100,000 g/mol, preferably from 40,000 to 70,000 g/mol.

Groups X and Z are defined as above.

The end groups of the linear statistical copolymer contain parts of polymerisation initiator molecules (e.g., azo initiator 2,2'-azobis(2-methylpropionitrile) (AIBN), 4,4′-azobis(4-cyanopentanoic acid) (ACVA), 2,2'-azobis(4-methoxy-2,4-dimethylpentanenitrile (V70)), and of a transfer agent selected from the group of 2-cyano-2-propylbenzodithioate, 4-cyano-4- (thiobenzoylthio)pentanoic acid, 2-cyano-2-propyldodecyl trithiocarbonate, 2-cyano-2-propylethyl trithiocarbonate and 4-cyano-4 -[(dodecylsulphanylthiocarbonyl)sulphanyl]pentanoic acid. Thus, in a preferred embodiment, the end groups of the copolymer contain at one end a radical leaving group of a transfer agent, preferably 2-cyano-2-propyl, or 5-carboxy-2-cyano-2-pentyl. At the other end there is a dithiobenzoate or trithiocarbonate group, which can be removed by reaction with excess AIBN or can be converted *in situ* after its reduction to other reactive groups, for example to dibenzocyclooctyne (DBCO), azide, carboxyl group, amino group, which are further usable for preparation of star copolymers.

In one embodiment, the polymeric conjugate is a star copolymer of general formula (IV),



(IV),

wherein the multivalent carrier is a second or third generation poly(amidoamine) (PAMAM) dendrimer (for example with ethylenediamine, 1,4-diaminobutane, 1,6-diaminohexane, 1,12-diaminododecane, cystamine core) or 2,2-bis(hydroxymethyl)propionic acid based second to fourth generation dendrimer or dendron (e.g. with trimethylolpropane nucleus);

X and Z are as defined above;

Y is selected from the group consisting of a primary amino group; alkyne group of 3 to 6 carbons, such as propargyl; and a cyclooctyne group which may be optionally further independently substituted with one or more groups selected from an alkyl group of 1 to 6 carbons and aryl of 6 carbons, e.g. DBCO;

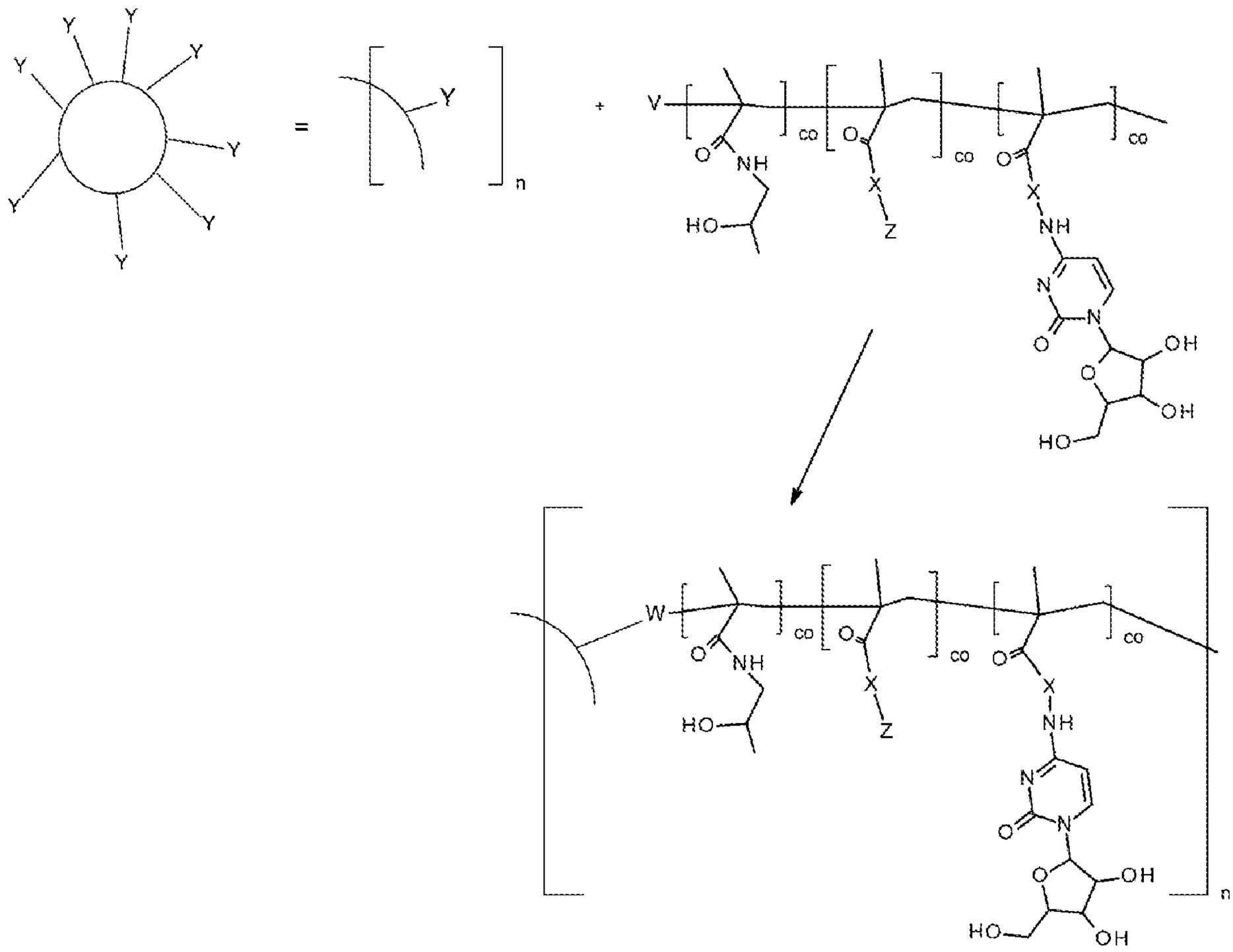
and W is an amide bond between the primary amino group Y of the multivalent carrier and the carboxyl end group of the polymer of general formula (III); or a triazole linker formed by the reaction of an azide end group of the polymer of general formula (III) and an alkyne or cycloalkyne group Y of a dendrimer or dendron (multivalent carrier).

Thus, the star copolymer consists of a central molecule of PAMAM dendrimer or 2,2-bis (hydroxymethyl)propionic acid-based dendrimer or dendron, to the end groups of which at least one chain of the linear statistical copolymer of general formula (III), as defined above, is attached. The end groups of the multivalent carrier are amino groups or alkyne groups of 3 to 6 carbons, e.g. propargyl, or cyclooctyne groups, which may be optionally further independently substituted with one or more groups selected from alkyl group of 1 to 6 carbons and aryl of 6 carbons, e.g. DBCO, and the linear copolymer of formula (III) is attached to them via an amide or triazole linker.

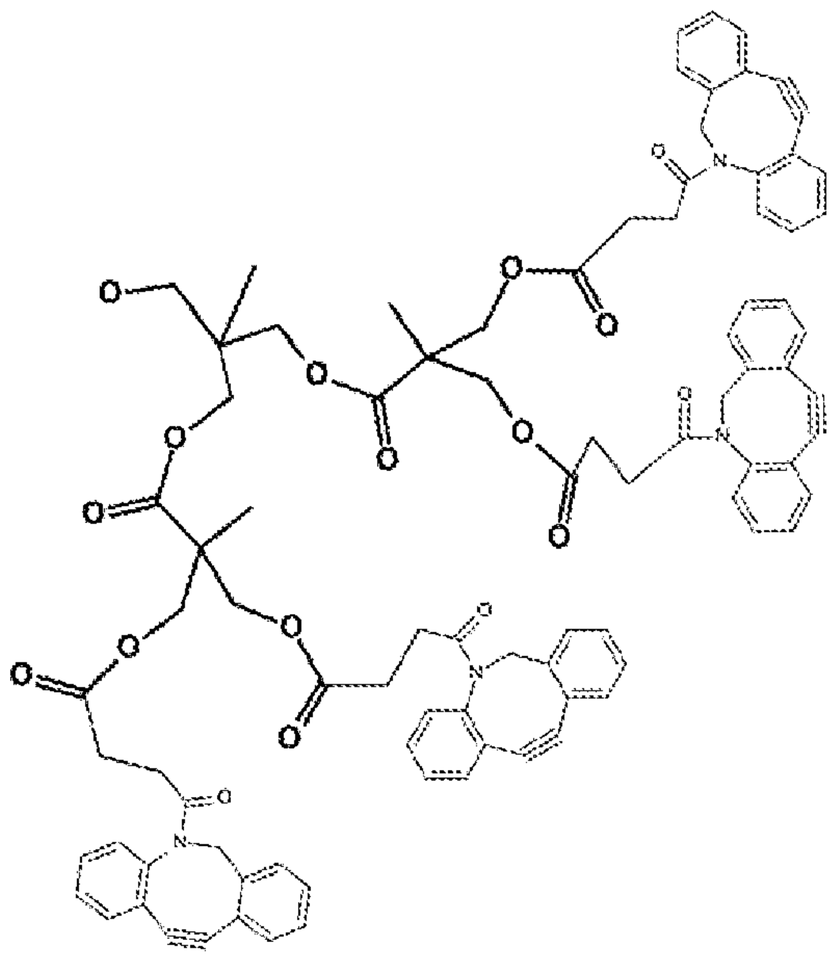
Preferably, the star copolymer comprises from 2 to 48 linked chains of the linear statistical copolymer of general formula (III), more preferably 8 to 32 linked chains of the linear statistical copolymer of general formula (III), most preferably 16 to 24 linked chains of the linear statistical copolymer of general formula (III).

Molar mass M_n of the star copolymer of general formula (IV) is in the range of from 60,000 to 1,000,000 g/mol, preferably from 100,000 to 400,000 g/mol. The molar mass of each chain of the linear statistical copolymer of general formula (III) bound to the multivalent carrier is from 4,000 to 100,000 g/mol, preferably 40,000 to 70,000 g/mol, while the molar mass of the multivalent carrier alone which is part of the copolymer of general formula (IV) does not exceed 50,000 g/mol. This ensures that after disintegration of the star copolymer into the multivalent carrier and the linear copolymer of general formula (III) in tumour tissue and release of cytarabine, all resulting fragments have a molecular weight below the renal filtration limit and are therefore well removable from the body by renal filtration.

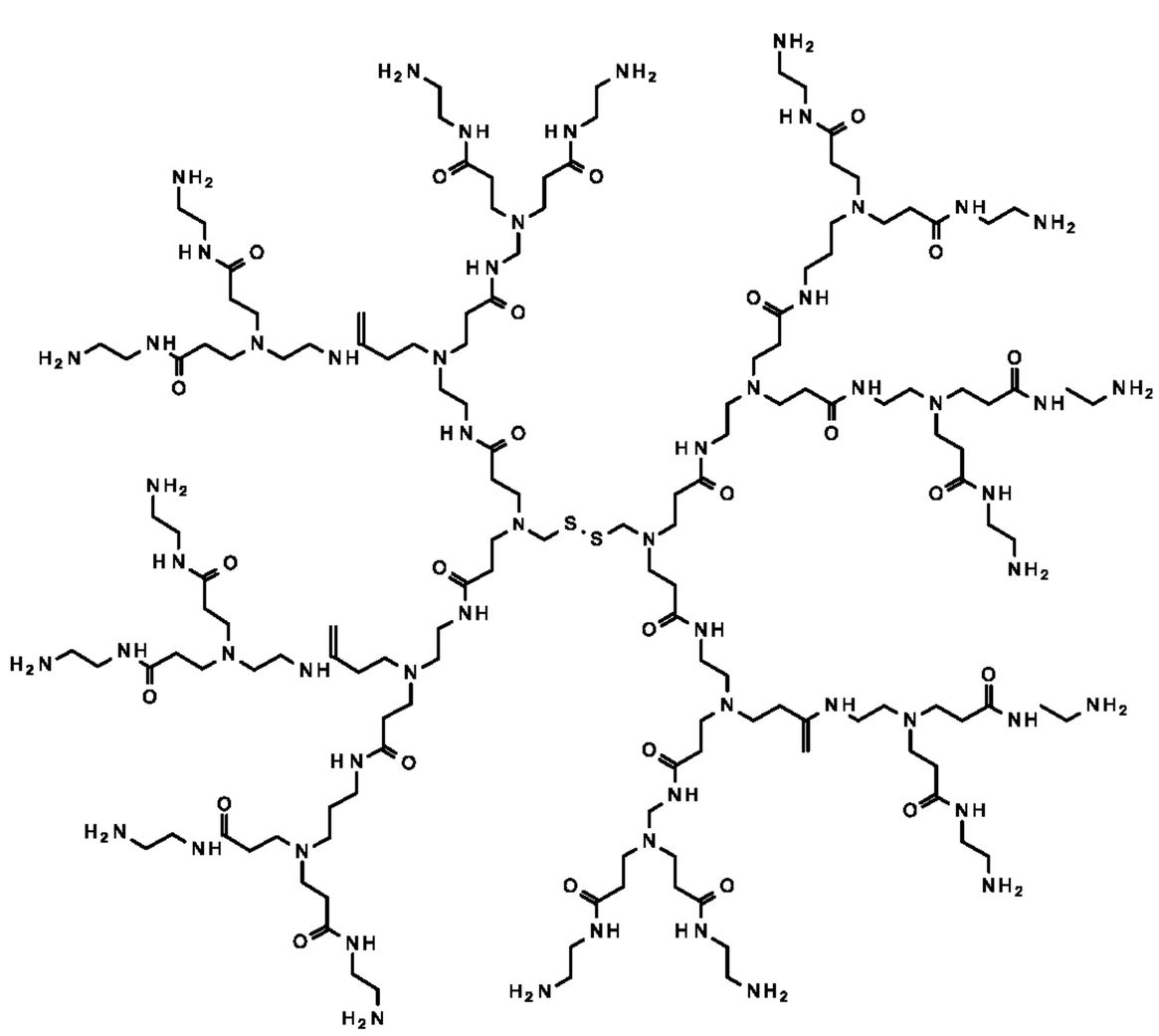
The method of preparation of the star copolymer of general formula (IV) is depicted in Scheme 1.



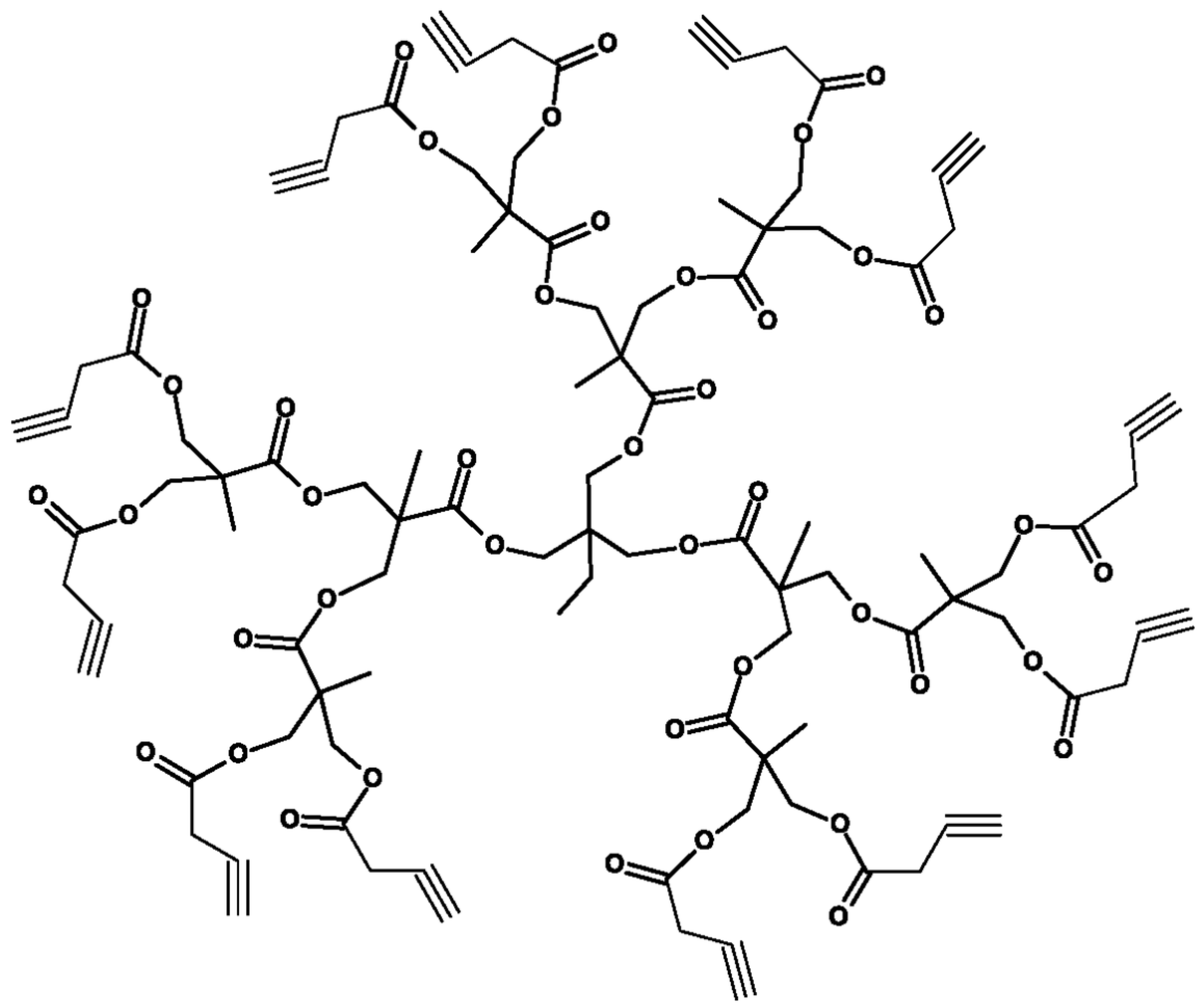
Scheme 1: Scheme of the preparation of a star copolymer, wherein *n* is an integer in the range of from 1 to 48, Y is a primary amino group, a C3-C6 alkyne group or a cyclooctyne group (e.g. DBCO group) of poly(amidoamine) or 2,2-bis(hydroxymethyl)propionic acid based dendrimer or dendron:
V is an azide or TT end reactive group introduced by a transfer agent to the end of the polymer chain of general formula (III); W is an amide bond or a triazole linker formed by the reaction of the general formula (III) polymer azide and an alkyne group or cycloalkynyl group of a dendrimer or dendron.



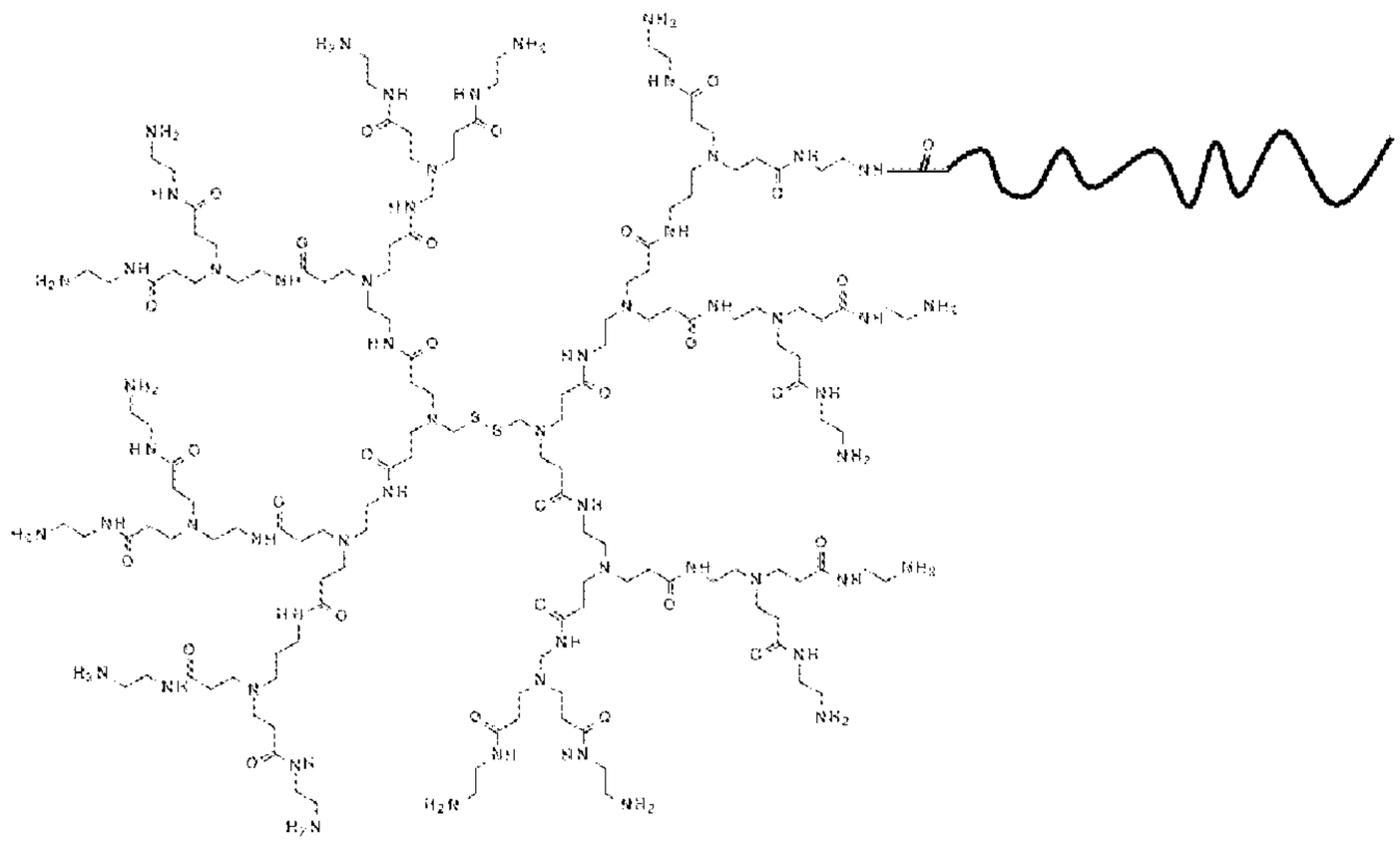
(i)



(ii)



(iii)



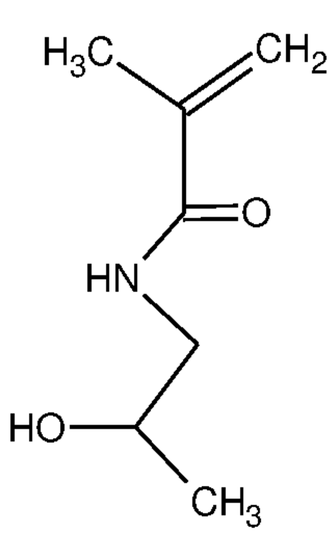
(iv)

Scheme 2: Examples of multivalent carrier structures for the synthesis of the star copolymer of general formula (IV): (i) schematic representation of bis-MPA dendron with DBCO groups, (ii) PAMAM

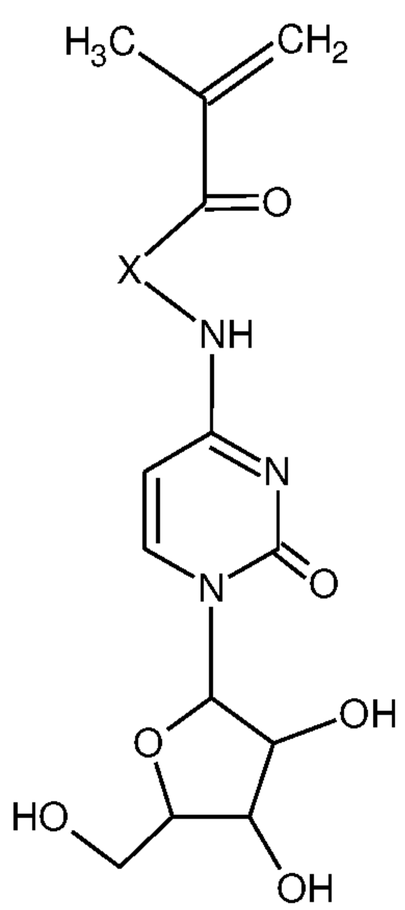
dendrimer with amino groups, (iii) bis-MPA dendrimer with propargyl groups and (iv) example of the structure of the star copolymer of general formula (IV) with PAMAM dendrimer carrier, in which the chains containing the linear statistical HPMA copolymer of formula (III) are marked schematically by a wavy line. The maximum number of chains of the linear copolymer that can be attached to the dendrimer is equal to the number of end groups of the dendrimer (in this case 16 end amino groups).

A further object of the present invention is a method for the preparation of the linear statistical copolymer of general formula (III) according to the present invention, which comprises the following steps:

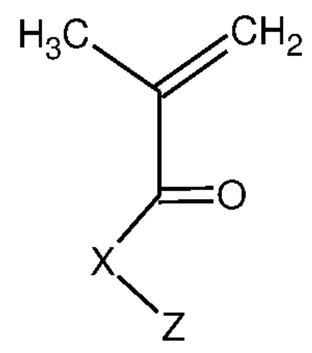
(i) providing an HPMA monomer and a monomer of general formula (V) and/or (VI)



HPMA

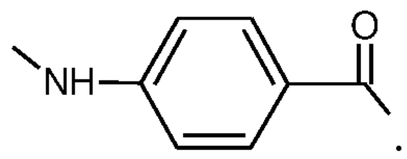


(V)



(VI);

wherein

X is a covalent bond or aminoacyl bonded by an amide bond to the carbonyl group of the monomeric unit (I) and optionally the monomeric unit (II) and selected from the group consisting of –NH–$(CH_2)_n$–C(=O)–, wherein *n* is an integer from 1 to 6; –NH–$(CH_2)_q$(C(=O)–NH–$(CH_2)_r)_p$–C(=O)–, wherein *p*, *q* and *r* are independently selected from 1, 2 and 3; 

;

–NH–$(CH_2)_n$–C(=O)–NH–N=C(A)–$(CH_2)_q$–L–C(=O)–; wherein *n* is an integer from 1 to 6; *q* is selected from 1, 2 and 3; A is selected from the group consisting of methyl, isopropyl, cyclohexyl and pyridyl; L is a covalent bond or 1,4-phenylene;

wherein –CH_2– of group X may be further substituted with one or more of the same or different natural amino acid side chains;

and/or the phenylene of group X may be further substituted with one or more of the same or different natural amino acid side chains;

preferably these natural amino acids chains are methyl, isopropyl, isobutyl, –$CH(CH_3)(CH_2CH_3)$, –CH_2OH, –$CH(OH)(CH_3)$, –CH_2–$(C_6H_4)OH$, –$(CH_2)_2$–S–CH_3, –CH_2SH, –$(CH_2)_4$–NH_2, –CH_2COOH, –$CH_2C(O)NH_2$, –$(CH_2)_2COOH$, –$(CH_2)_2C(O)NH_2$, –$(CH_2)_3NH$–$C(=NH)(NH_2)$, benzyl;

with the proviso that X is not –NH–$(CH_2)_2$–C(=O)– and that X is not –NH–CH_2–C(=O)–NH–$CH(CH_2$–Ph)–C(=O)–NH–$CH(CH_2$–iPr)–C(=O)–NH–CH_2–C(=O)–;

and Z is a reactive substitution derivative of X as defined above, preferably Z is selected from the group comprising –COOH; thiazoline-2-thione (TT); (4-nitrophenyl)oxy group; (2,3,4,5,6-pentafluorophenyl)oxy group; (succinimidyl)oxy group; –OH; Boc–NH–NH– (tert-butyloxycarbonyl hydrazide);

(ii) the step of radical polymerisation of HPMA with monomers of the general formula (V) and/or (VI);

(iii) in case the step (ii) is the polymerisation of HPMA with a monomer of general formula (VI), the step of attaching cytarabine to the Z group of the polymer of step (ii), as defined above;

(iv) removal of the end reactive groups of the linear copolymer.

Ad (i) The provision of HPMA monomers and monomers of general formula (V) and/or (VI) comprises the provision of *N*-(2-hydroxypropyl)methacrylamide (HPMA), which is commercially available. Monomers of general formula (V) are prepared by reacting the corresponding monomer of general formula (VI) with cytarabine in pyridine at elevated temperature for at least 48 h. Monomers of general formula (VI) are prepared by acylation of the corresponding amino acids (from which aminoacyls X described above are derived) or derivatives thereof with reactive methacrylic acid derivatives, preferably with methacroyl chloride, and subsequent modification of the end aminoacyl

with the reactive group Z defined above in a non-aqueous medium with using carbodiimide-based condensing agents.

Ad (ii) The radical polymerisation takes place at a temperature in the range of from 30 to 100 °C, preferably from 40 to 80 °C, in a solvent preferably selected from the group consisting of water, aqueous buffers, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol, dioxane, *tert*-butyl alcohol or mixtures thereof, initiated by an initiator, preferably selected from the group comprising, in particular, azo initiators 2,2′-azobis(2-methylpropionitrile) (AIBN), 4,4′-azobis(4-cyanopentanoic acid) (ACVA), 2,2'-azobis(4-methoxy-2,4-dimethylpentanenitrile) (V70), optionally in the presence of a transfer agent, preferably selected from the group comprising 2-cyano-2-propylbenzodithioate, 4-cyano-4-(thiobenzoylthio)pentanoic acid, 2-cyano-2-propyldodecyl-trithiocarbonate, 2-cyano-2-propylethyltrithiocarbonate and 4-cyano-4-[(dodecylsulphanylthio-carbonyl)sulphanyl] pentanoic acid. The molar mass M_n of the linear copolymers thus prepared is in the range of from 4,000 to 100,000 g/mol, preferably from 30,000 to 50,000 g/mol.

Ad (iii) The optional reaction of the monomeric unit of general formula (II) with cytarabine takes place at a temperature between 20 and 100 °C, preferably from 30 to 60 °C in a solvent preferably selected from the group comprising pyridine, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol or mixtures thereof. The molar mass M_n of the linear conjugates thus prepared is in the range of from 4,000 to 100,000 g/mol, preferably from 40,000 to 70,000 g/mol. When the monomeric unit of general formula (II) contains a *tert*-butyloxycarbonyl hydrazide group, the reaction with cytarabine is preceded by removal of the Boc protecting group, for example by acid hydrolysis with HCl or CF_3COOH, or by thermal hydrolysis in water at 100 °C.

Ad (iv) The step of removing the end reactive groups of the linear copolymers in the preparation of which the transfer agent was used. In this step, the end groups of the linear polymer of step (ii) or (iii) are reacted with an excess of azo initiator selected from the group of initiators described in step (ii) at a temperature in the range of from 50 to 100 °C, preferably from 60 to 80 °C, in a solvent preferably selected from the group comprising dimethylsulphoxide, dimethylacetamide and dimethylformamide, to form the polymer of general formula (III).

Another object of the present invention is a method for the preparation of the star copolymer as defined above, comprising the following steps:

(i) Providing an HPMA monomer and a monomer of general formula (V) and/or (VI) which is the same as in the process for preparing the linear copolymer (III) of the present invention;

(ii) Radical copolymerisation of HPMA with monomers of general formula (V) and/or (VI) at a temperature in the range of from 30 to 100 °C, preferably from 40 to 80 °C, in a solvent preferably selected from the group comprising water, aqueous buffers, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol, dioxane, *tert*-butyl alcohol or mixtures thereof, initiated by an initiator, preferably selected from the group comprising, in particular, azo initiators 2,2′-azobis(2-methylpropionitrile) (AIBN), 4,4′-azobis(4-cyanopentanoic acid) (ACVA), 2,2'-azobis (4-methoxy-2,4-dimethylpentanenitrile) (V70), in the presence of a transfer agent containing reactive functional groups allowing grafting of the dendrimer or its derivative prepared by the prepared semitelechelic copolymer. The transfer agent is selected from the group comprising [4-(3-azidopropylamino)-1-cyano-1-methyl-4-oxo-butyl]benzenecarbodithioate, N-(3-azidopropyl)-4-cyano-4-ethylsulphanyl-carbothioylsulphanyl-pentanamide, [1-cyano-1-methyl-4-oxo-4-(2-thioxothiazolidin-3-yl)butyl] benzenecarbodithioate or 2-ethylsulphanylcarbothioyl-sulphanyl-2-methyl-5-oxo-5-(2-thioxo-thiazolidin-3-yl)pentanenitrile. The molar mass M_n of the linear copolymers thus prepared is in the range of from 4,000 to 100,000 g/mol, preferably from 25,000 to 50,000 g/mol. The resulting semitelechelic linear copolymer of general formula (III) contains end reactive groups (azide and TT) introduced by the transfer agent are further used in step (iii).

(iii) Providing a multivalent carrier for grafting the semitelechelic copolymer of step (ii), wherein the multivalent carrier is selected from the group comprising second or third generation poly (amidoamine) (PAMAM) dendrimer and 2,2-bis(hydroxymethyl) propionic acid (bis-MPA) based second, third or fourth generation dendrimer or dendron, terminated by Y groups selected from primary amino groups, alkyne groups of 3 to 6 carbons, e.g. propargyl, and cyclooctyne groups which may be optionally further independently substituted with one or more groups selected from an alkyl group of 1 to 6 carbons and aryl of 6 carbons, e.g. DBCO.

Said multivalent carriers are commercially available, optionally the DBCO end groups can be prepared by reacting primary amino end groups with DBCO-NHS.

(iv) Step of grafting the reactive azide or TT functional groups of the linear semitelechelic copolymer of general formula (III) prepared in step (ii) to the Y groups of the multivalent carrier from step (iii) to form a star copolymer containing in its arms HPMA monomeric units, units of general formula (I) and, where appropriate, of general formula (II). The grafting reaction of the polymers onto the cores takes place in a solvent preferably selected from the group comprising dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol and ethanol. The molar mass M_n of the copolymers thus prepared is in the range of from 60,000 to 1,000,000 g/mol, preferably from 100,000 to 400,000 g/mol.

(v) Optionally, if the resulting star copolymer of step (iv) contains monomeric units of general formula (II), the step of attaching cytarabine to the Z group of the polymer of step (iv) at a temperature between 20 and 100 °C, preferably from 30 to 60 °C, in a solvent preferably selected from the group comprising pyridine, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol or mixtures thereof. When the monomeric unit of general formula (II) contains a *tert*-butyloxycarbonyl hydrazide group, the reaction with cytarabine is preceded by removal of the Boc protecting group, for example by acid hydrolysis with HCl or CF_3COOH, or by thermal hydrolysis in water at 100 °C.

Another object of the present invention is a pharmaceutical composition comprising as active ingredient the linear statistical polymeric conjugate of general formula (III) according to the present invention and/or a star polymeric conjugate of general formula (IV) according to the present invention; wherein the pharmaceutical composition further comprises at least one pharmaceutically acceptable excipient selected from the group comprising anti-adhesives, binders, coating agents, colourants, swelling agents, flavouring agents, lubricants, preservatives, sweeteners, sorbents. For injectable dosage form of administration of the pharmaceutical preparation, most often as a bolus or as an infusion, preferably intravenously, suitable pharmaceutically acceptable excipients are solvents (especially water or saline), buffers (especially phosphate buffer, HEPES = 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulphonic acid), ionising additives, antioxidants, anti-microbial additives. A person skilled in the art would be able to determine which pharmaceutically acceptable excipient to choose without performing the inventive activity.

Another object of the present invention is the use of the polymeric conjugate of cytarabine as defined above in human medicine, in particular in the treatment of malignancies, preferably for the treatment of solid tumours and/or haematological tumours, more preferably for the treatment of leukaemias and non-Hodgkin's lymphomas.

The polymeric conjugate of cytarabine according to the present invention, in which cytarabine is bound to the polymeric carrier by an amide bond to the carbonyl group of group X as defined above, does not allow deamination of cytarabine in plasma after drug administration, and thus disables its degradation to uracil derivative. For this reason, the circulation time of bound cytarabine in the body is significantly prolonged, which makes it possible to transport cytarabine on high-molecular polymer carrier to the site of action and to release it there in its original form.

The polymeric drug according to the present invention is further characterized in that the binding of the drug to the polymeric carrier is relatively stable, so that a maximum of 7 % of the drug is released in 24 h during transport in the bloodstream and body fluids, and the binding is also hydrolytically cleavable in the tumour environment and within target tumour cells in lysosomes. This means that the drug is transported through the bloodstream in an inactive, polymer-bound form, and its release and activation occurs mainly after entering the tumour tissue or penetrating the target tumour cells. Activation of the drug only in the target cells leads to the protection of cytarabine against premature degradation in the organism, elimination of side effects and targeting of its effect preferentially on tumour cells. The binding of cytarabine to the polymeric carrier significantly increases the molecular weight of the drug and thus prolongs its circulation in the bloodstream in an inactive form, prolongs the total residence time of the drug in the body and thus increases its bioavailability. The targeted (passive) transport to the tumour or tumour cells is the responsibility of the polymeric carrier prepared on the basis of HPMA copolymers, the molecular weight and thus the efficiency of accumulation in tumour tissue of which can be controlled by changes in the structure of the polymeric carrier (linear polymer, high- molecular-weight biodegradable star polymer). Due to the increased molecular weight of the polymeric carrier, the whole conjugate is accumulated through the EPR effect in solid tumours and also in non-Hodgkin's lymphomas, where cytarabine is activated by its release from the polymeric prodrug. Cytarabine is released in tumour tissue mainly due to spontaneous hydrolysis of the amide bond, but also due to the lysosomal enzymes of tumour cells present. Preferably, the linear polymeric

precursor has a molecular weight below the limit of renal filtration, and is thus easily removed from the body by renal filtration. After cytarabine binding, the molecular weight increases above the limit of renal filtration and thus there is a significant prolongation of the circulation time and accumulation in solid tumours and lymphomas. Upon release of cytarabine, the molecular weight of the polymeric carrier decreases again to a value below the limit of renal filtration.

Synthesis and structures of polymeric conjugates

The synthesis of the polymeric conjugates of the invention is performed in several steps, the detailed final structure of the conjugate significantly depending on the chosen synthetic route (see above).

In the first step of the synthesis, the **basic monomers** are synthesized: HPMA, monomers of general formulas (V) and (VI), which are methacryloylated derivatives of amino acids and oligopeptides terminated by an amino-reactive group, methacryloylated derivatives of amino acids and oligopeptides with amide-linked cytarabine.

In the second step, precursors, i.e. linear statistical copolymers of HPMA and monomeric units of general formulas (I) and/or (II), bearing functional groups serving as polymeric drug carriers, or cytarabine polymeric conjugates themselves, are synthesized by radical polymerisation.

The polymeric precursor bearing an amino-reactive functional group along the chain can be prepared either by radical copolymerisation of the above-mentioned functional monomers with HPMA or by their controlled radical (RAFT) polymerisation.

A polymeric precursor is copolymer of HPMA and methacryloylated amino acids or oligopeptides, or hydrazides of amino acids selected from the group consisting of aminomethanoyl (AM), 2-aminoethanoyl (AE), 6-aminohexanoyl (AH), 3-aminopropanoyl (AP), 4-aminobutanoyl (AB), 5-aminopentanoyl (VAL), glycylglycyl (GG), glycyl (Gly), 4-aminobenzoyl (BA) and acyls derived from oligopeptides of 2 to 3 amino acids, characterised in that it contains 80 to 95 mol% of HPMA and 5 to 20 mol% of units with amino-reactive functional groups.

A polymeric conjugate is a compound of the polymeric precursor with cytarabine or its derivative with an oxo acid on the amino group, where cytarabine is attached to the polymeric precursor by an amide bond prepared by reacting the amino group of the drug with amino-reactive groups of the polymer, or prepared by reacting the amino group of the drug with amino-reactive groups of the

monomer and subsequent copolymerization with HPMA, or cytarabine is first modified with an oxo acid to form an amide bond between cytarabine and oxo acid, levulinic acid (LEV), 4-pyridyl-4-oxo-butanoic acid (PYR), 4-2-oxopropyl-benzoic acid (OPB), 5-cyclohexyl-5-oxo-pentanoic acid (COP) and 5-methyl-4-oxo-hexanoic acid (ILE), and subsequently the oxo group of the derivative is used to bind via a hydrazone bond to the polymeric precursor with hydrazide groups which contains 80 to 95 mol% of HPMA and at least 5 mol% of units with amide-linked cytarabine.

Brief description of drawings

Figure 1: Graph showing the rate of cytarabine release from linear polymeric conjugates in pH 7.4 buffer (bloodstream model).

Figure 2: Graph showing the rate of cytarabine release from linear polymeric conjugates in pH 7.4 buffer (bloodstream model) and pH 5 buffer (cell model).

Figure 3: Demonstration of in vivo biological activity. Polymeric araC conjugates are more effective in treating MCL compared to araC alone. The following dosages were used: araC 4 mg/mouse (rhomb), **polymeric conjugate** p(HPMA-*co*-Ma-GFLG-araC) 4 mg/mouse (cross), **polymeric conjugate** p(HPMA-*co*-MA-AH-araC) 4 mg/mouse (circle).

Examples

Examples of the synthesis of intermediates and conjugates according to the invention

Example 1: Synthesis of monomers

HPMA was prepared according to the procedure previously described (Ulbrich K, Šubr V, Strohalm J, Plocová D, Jelínková M, Říhová B, Polymeric Drugs Based on Conjugates of Synthetic and Natural Macromolecules I. Synthesis and Physico-chemical Characterisation. J. Controlled Rel. 64, 63-79 (2000)). Elemental analysis: calculated 58.8 % C, 9.16 % H, 9.79 % N; found 58.98 % C, 9.18 % H, 9.82 % N. The product was chromatographically pure.

3-(6-Methacrylamidohexanoyl)thiazolidine-2-thione (MA-AH-TT) was prepared according to the procedure previously described by Šubr V, Ulbrich K, Synthesis and properties of new N-(2-hydroxypropyl)-methacrylamide copolymers containing thiazolidine-2-thione reactive, Reactive & Functional Polymers 66, 1525–38 (2006).

3-(2-Methacrylamidoglycyl)thiazolidine-2-thione (MA-G-TT),

3-(methacrylamidoglycylglycyl)thiazolidine-2-thione (MA-GG-TT),

3-(4-methacrylamidobenzoyl)thiazolidine-2-thione (MA-BA-TT),

3-(5-methacrylamidopentaoyl)thiazolidine-2-thione (MA-VAL-TT) were prepared according to the same procedure as MA-AH-TT.

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidohexanoyl)hydrazine (MA-AH-NHNH-Boc) was prepared according to the procedure previously described (K. Ulbrich, T. Etrych, P. Chytil, M. Jelinkova and B. Rihova, *J Drug Target*, 2004, **12**, 477-489).

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidometanoyl)hydrazine (MA-AM-NHNH-Boc),

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidoetanoyl)hydrazine (MA-AE-NHNH-Boc),

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidopropanoyl)hydrazine (MA-AP-NHNH-Boc),

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidobutanoyl)hydrazine (MA-AB-NHNH-Boc),

1-(*tert*-butoxycarbonyl)-2-(6-methacrylamidopentanoyl)hydrazine (MA-VAL-NHNH-Boc) were prepared according to the same procedure as MA-AH-NHNH-Boc.

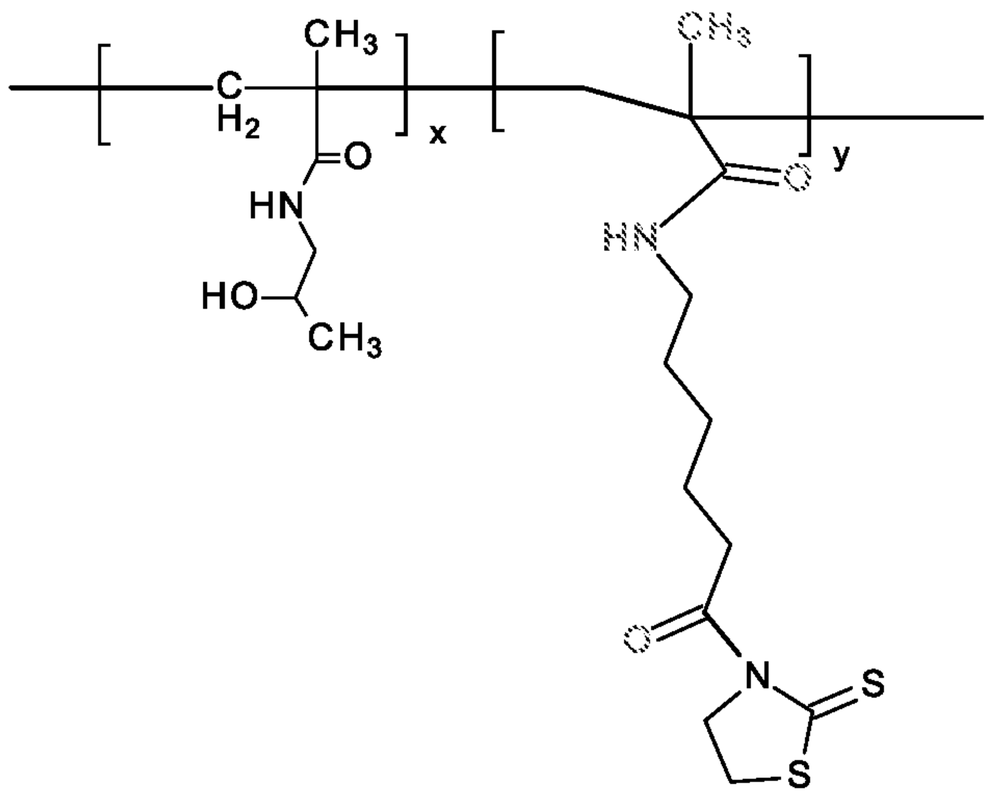
Monomer with MA-AH-araC was prepared by reacting said reactive monomer Ma-AH-TT according to the following procedure. MA-AH-TT (100 mg) was dissolved together with 81 mg of cytarabine in 1 mL of pyridine under Ar atmosphere at 50 °C and stirred for 3 days. The solution was then precipitated into diethyl ether. The product was precipitated several times from methanol into diethyl ether and characterized by HPLC and NMR. From the measured UV spectrum (by Helios Alpha UV/vis 135 instrument, Thermospectronic, UK) of this monomer of known concentration, the molar absorption coefficient in methanol was calculated at 301 nm ε_{301} = 8,000 $l \cdot mol^{-1} \cdot cm^{-1}$ used further to determine the content of cytarabine in polymeric conjugates. The value of the coefficient thus determined corresponded to the value of the coefficient obtained from the cytarabine content determined by 1H NMR analysis of the polymeric conjugates.

The purity of all monomers was determined using the HPLC system [Shimadzu HPLC system equipped with the Chromolith Performance RP-18e reverse phase column (100 × 4.6 mm) and Shimadzu SPD-10AVvp UV-VIS detector (230 nm); eluent water – acetonitrile with a gradient of 0–100 vol.% of acetonitrile, flow rate 5 $mL \cdot min^{-1}$.

Example 2: Synthesis of cytarabine polymeric conjugate by direct copolymerisation

The polymeric conjugate poly(HPMA-co-MA-AH-araC) was prepared by controlled solution radical copolymerisation of HPMA (95 mol%, 100 mg) and MA-AH-araC (5 mol%, 15.6 mg) in methanol at 60 °C in the presence of AIBN initiator 4-cyano-4-thiobenzoylsulphanylpentanoic acid as transfer agent. The polymerisation mixture was dissolved in tert-butyl alcohol and transferred to a glass ampoule to be bubbled with Ar and sealed. After 16 h at 70 °C, the polymer was isolated by precipitation into acetone. The precipitate was then washed with diethyl ether and dried in vacuum. The end dithiobenzoate groups were removed from the copolymer by reaction with AIBN (10-fold molar excess) in DMSO (15% polymer solution) under argon atmosphere for 3 h at 70 °C in a sealed vial. The polymeric conjugate was isolated by precipitation into acetone. The precipitate was washed with diethyl ether and dried in vacuum to dryness. The prepared conjugate had M_w= 45,000 g/mol, $Đ$= 1.17, R_h = 4.2 nm, cytarabine content 9.5 wt%. All other polymeric conjugates containing other amino acid or oligopeptide linkers, poly(HPMA-co-MA-BA-araC), poly(HPMA-co-MA-G-araC), poly(HPMA-co-MA-GG-araC) were prepared analogously to this procedure.

Example 3: Synthesis of polymeric precursor - copolymer of HPMA with MA-AH-TT



The copolymer poly (HPMA-co-MA-AH-TT) was prepared by controlled RAFT solution radical copolymerization of HPMA and MA-AH-TT in methanol at 60 °C according to the same procedure as described in Example 2. Other polymeric precursors, poly(HPMA-co-MA-BA-TT), poly(HPMA-co-MA-G-TT), poly(HPMA-co-MA-VAL-TT) and poly(HPMA-co-MA-GG-TT) containing other amino acid or oligopeptide linkers were prepared analogously to this procedure. The polymeric

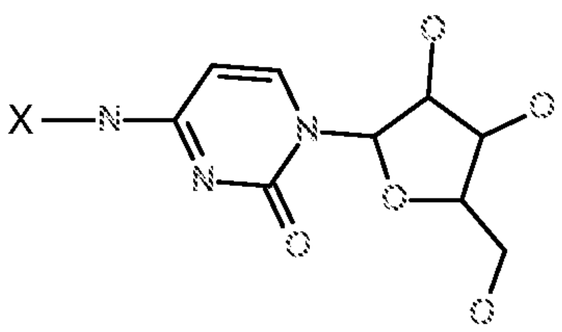
precursors had molecular weights below the renal filtration limit for pHPMA copolymers, determined to be 50,000 g/mol, which subsequently allows the carrier itself to be removed after drug delivery without the risk of its accumulation in the body.

Table 1 Characterization of prepared polymeric precursors

Precursor	**M_W** g/mol	**M_W/M_n**	**R_h** nm	**TT content** mol %
p(HPMA-*co*-MA-BA-TT)	43,000	1.16	4.6	9.8
p(HPMA-*co*-MA-AH-TT)	44,500	1.12	4.3	11.3
p(HPMA-*co*-MA-GG-TT)	42,000	1.15	4.5	11.8
p(HPMA-*co*-MA-G-TT)	40,000	1.12	4.4	10.9
p(HPMA-co-MA-VAL-TT)	34,500	1.08	4.5	10.0

Example 4 Synthesis of the oxo-derivative of cytarabine – Lev-Cyt

Lev-Cyt derivative was prepared by modification of cytarabine with activated levulinic acid. Cytarabine (18.0 mg, 74 μmol) and Lev-TT (19.8 mg, 74 μmol) were dissolved in 0.35 mL of pyridine. The reaction mixture was bubbled with argon and the reaction was run at 50 °C. The course of reaction was monitored by HPLC. After 2 days the reaction was complete, the solution was precipitated into ethyl ether and the product was dried, characterised by ESI MS (364 g/mol M + Na^+) and HPLC. Other cytarabine derivatives with oxo-acids, Pyr-Cyt with 4-pyridyl-4-oxo- butanoic acid, Opb-Cyt with 4-2-oxopropyl benzoic acid, Cop-Cyt with 5-cyclohexyl-5-oxo- pentanoic acid and Ile-Cyt with 5-methyl-4-oxo-hexanoic acid, were prepared in the same manner.



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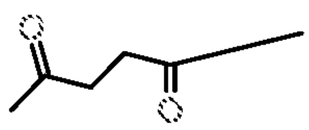
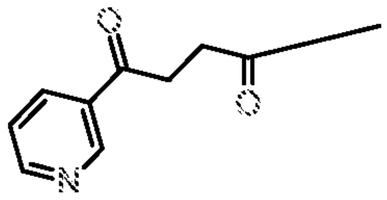
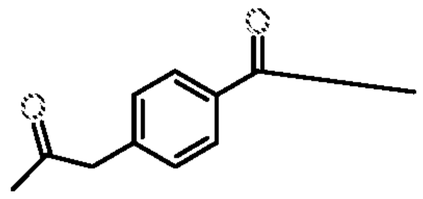


Lev



Pyr



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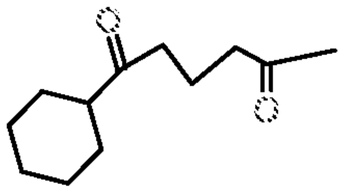

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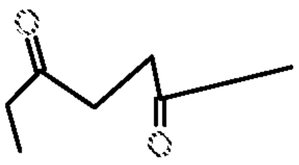

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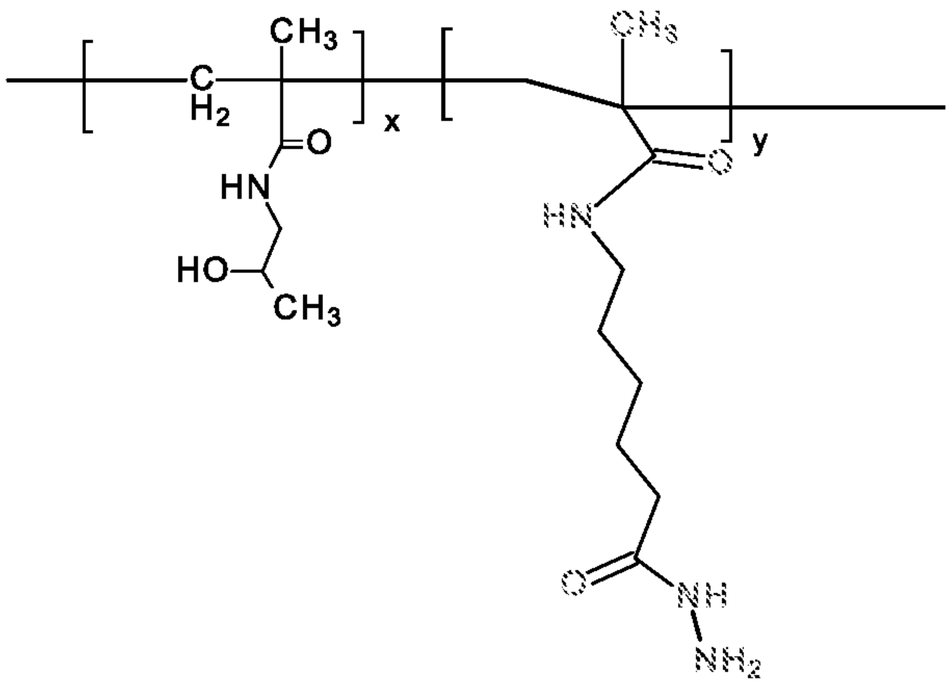
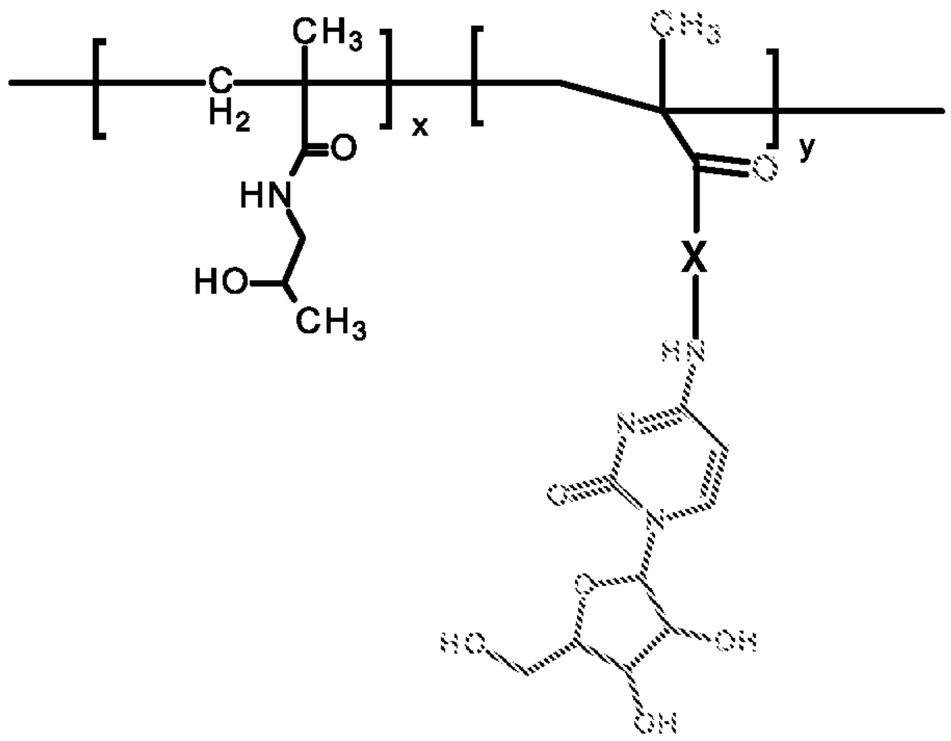
Example 5 Synthesis of polymeric precursor - copolymer of HPMA with MA-AH-NH-NH_2



The copolymer poly(HPMA-co-Ma-AH-NH-NH_2) was prepared by a two-step synthesis. In the first step, the copolymer poly(HPMA-co-MA-AH-NH-NH-Boc) was prepared by controlled RAFT solution radical copolymerisation of HPMA and MA-AH-NHNH-Boc in methanol at 60 °C according to the same procedure as described in Example 2. The polymeric precursor poly(HPMA-co-MA-AH-NH-NH_2) was prepared from HPMA-co-MA-AH- NH-NH-Boc by removing Boc protecting groups by incubation in water at 100 °C for 30 min. The final product was obtained by lyophilisation. Other polymeric precursors, poly(HPMA-co-Ma-AM-NH-NH_2), poly(HPMA-co-Ma-AE-NH-NH_2), poly(HPMA-co-Ma-AP-NH-NH_2), poly(HPMA-co-Ma-AB-NH-NH_2) and poly(HPMA-co-Ma-VAL-NH-NH_2) were prepared analogously to this procedure. The polymeric precursors had molecular weights below the renal filtration limit for pHPMA copolymers, determined to be 50,000 g/mol, which subsequently allows the carrier itself to be removed after drug delivery without the risk of its accumulation in the body.

Example 6 Preparation of polymeric conjugates containing the drug cytarabine by a polymer analogous reaction



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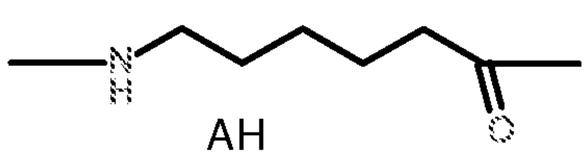
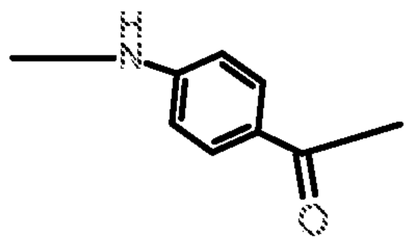


AH



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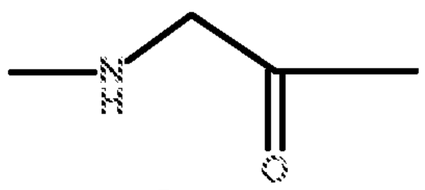

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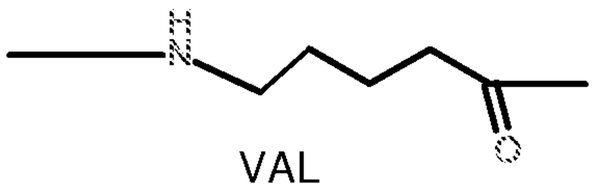
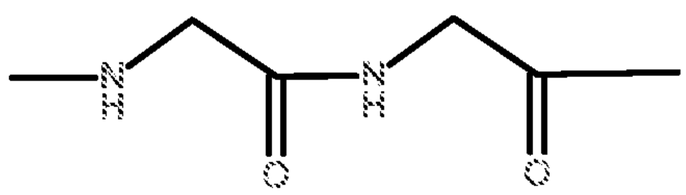


VAL



GG

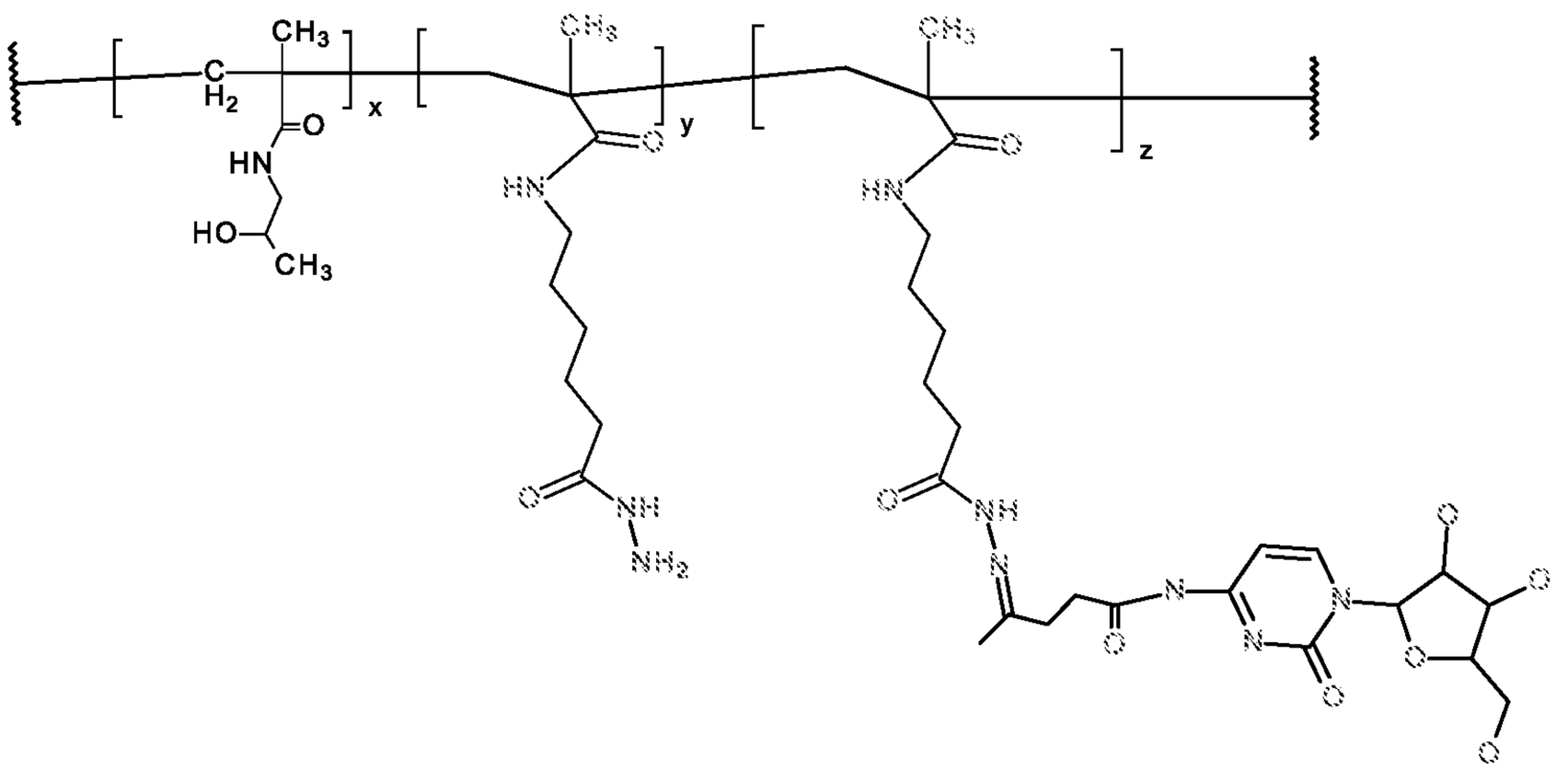
Polymeric conjugates of cytarabine with various linkers attached to a PHPMA carrier by a hydrolytically cleavable bond were prepared by reacting polymeric precursors containing amino-reactive groups with cytarabine in pyridine at 50 °C for 3 days. The polymeric conjugate poly(HPMA-*co*-MA-AH-araC) was prepared according to the following procedure: 850 mg of copolymer poly(HPMA-*co*-MA-AH-TT) and 150 mg of cytarabine was dissolved in 10 mL of pyridine and the solution was bubbled with argon and stirred at 50 °C for 3 days. Subsequently, the colourless solution was precipitated into diethyl ether, and the precipitate was dried and purified by gel filtration from free drug on a column packed with Sephadex G-25 in water. The polymeric fraction was isolated and lyophilised. The content of total cytarabine in the polymeric conjugate was determined by UV/Vis spectrophotometry as described in Example 1, Mw and molecular weight distribution and hydrodynamic radius R_h were determined by liquid chromatography (TSKGel 3000 column (300x10 mm), 20% 0.3 M acetate buffer (CH_3COONa/CH_3COOH; pH 6.5; 0.5 g/L NaN_3) and 80% methanol, flow rate 0.5 mL/min, detection by differential refractometer, light scattering detector (DAWN-DSP-F, Wyatt Technology, USA) and UV detector (250 nm). The characterisation of the polymeric drug is

given in Table 2. Total yield of drug binding reaction: 860 mg (86 %). The procedure for binding cytarabine to polymeric precursors with other linkers was similar. The molecular weights of the polymeric conjugates are just above the limit of renal filtration, which should allow a prolonged circulation of these polymeric cytarabine conjugates and thus a greater accumulation within the lymphomatous tissue. Upon drug release, these polymeric carriers with molecular weights below the limit of renal filtration should be well excretable from the body.

Table 2 Characterisation of polymeric conjugates

Precursor	**M_W** g/mol	**M_W/M_n**	**R_h** nm	**Cytarabine content** wt %
p(HPMA-*co*-MA-BA-araC)	62,800	1.35	5.2	13.2
p(HPMA-*co*-MA-AH-araC)	61,800	1.32	5.4	14.2
p(HPMA-*co*-MA-GG-araC)	63,300	1.35	5.5	13.9
p(HPMA-*co*-MA-G-araC)	62,600	1.39	5.3	13.5
p(HPMA-*co*-MA-VAL-araC)	53,100	1.30	5.3	13.1

Example 7 Preparation of polymeric conjugates containing the drug cytarabine attached by a combination of hydrolytically cleavable bonds



To 100 mg of the polymeric precursor poly(HPMA-co-MA-AH-NH-NH_2) prepared in Example 5, 15 mg of ***Lev-Cyt***, the oxo acid-modified cytarabine of Example 4, was bound in DMA in the presence

of acetic acid (100 mg of polymer / 40 µl CH_3COOH/1 mL of DMA). The course of reaction was monitored by TLC (methanol : $CHCl_3$ (3: 1)). After binding of all cytarabine, the polymerisation mixture was precipitated into diethyl ether and the precipitate was centrifuged. The product, p(HPMA-*co*-MA-AH-hydr-Lev-araC), was purified by gel chromatography on a column packed with Sephadex LH-20 in water. The polymeric fraction was isolated and lyophilised. The content of total cytarabine in the polymeric conjugate was determined by UV/Vis spectrophotometry as described in Example 1. Mw and molecular weight distribution and hydrodynamic radius R_h were determined by liquid chromatography as described in Example 3. Polymeric conjugates p(HPMA-*co*-MA-AH-hydr-OPB-araC), p(HPMA-*co*-MA-AH-hydr-PYR-araC) and p(HPMA-*co*-MA-AH-hydr-ILE-araC) were prepared analogously. The characteristics of all prepared polymeric conjugates are given in Table 3.

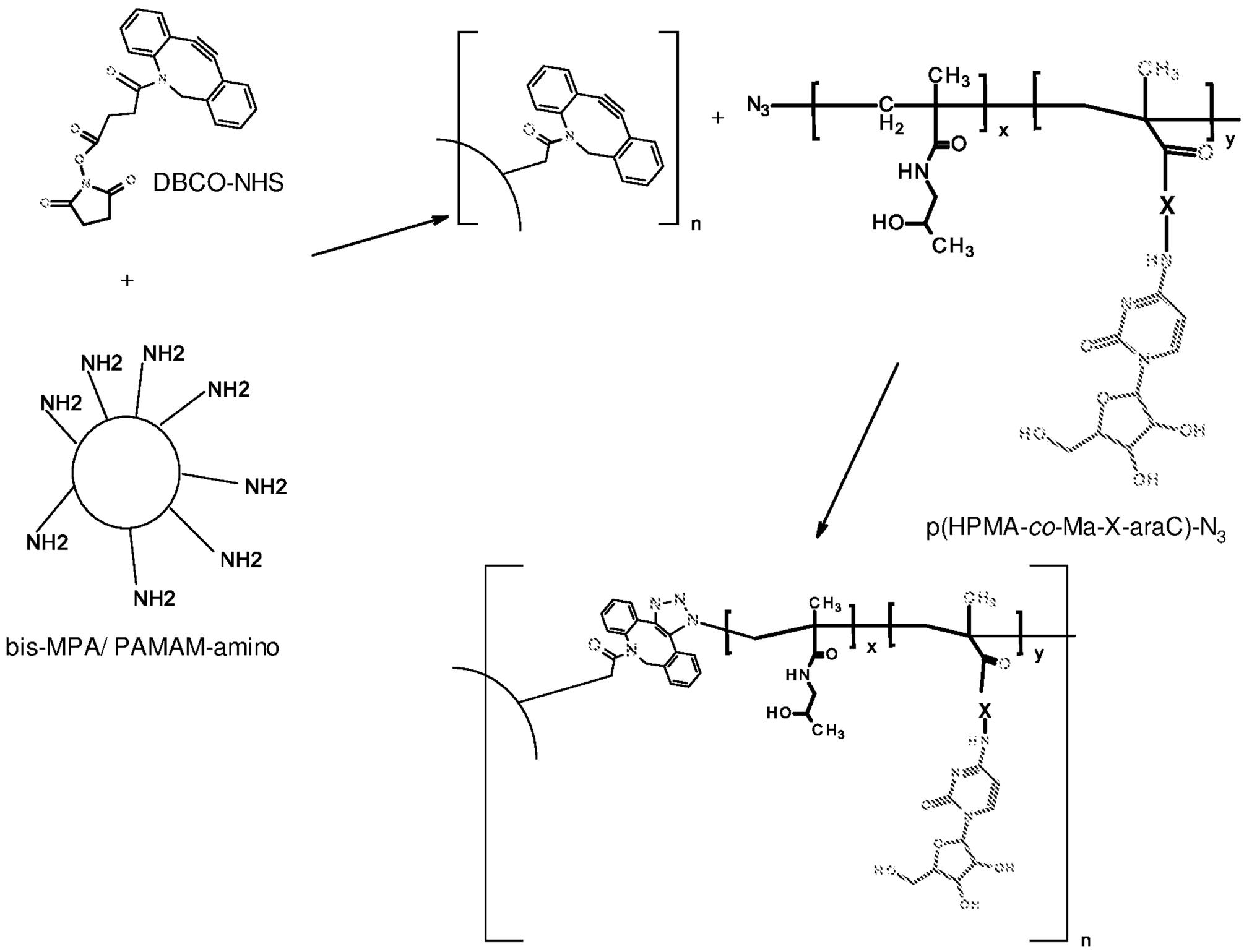
Table 3 Characterisation of the polymeric conjugates of Example 7

Precursor	**M_W** g/mol	**M_W/M_n**	**R_h** nm	**Cytarabine content** wt %
p(HPMA-*co*-MA-AH-hydr-LEV-araC)	42,500	1.45	5.3	12.2
p(HPMA-*co*-MA-AH-hydr-OPB-araC)	41,600	1.38	5.2	11.2
p(HPMA-*co*-MA-AH-hydr-PYR-araC)	42,300	1.39	5.5	12.8
p(HPMA-*co*-MA-AH-hydr-ILE-araC)	42,600	1.42	5.5	13.4

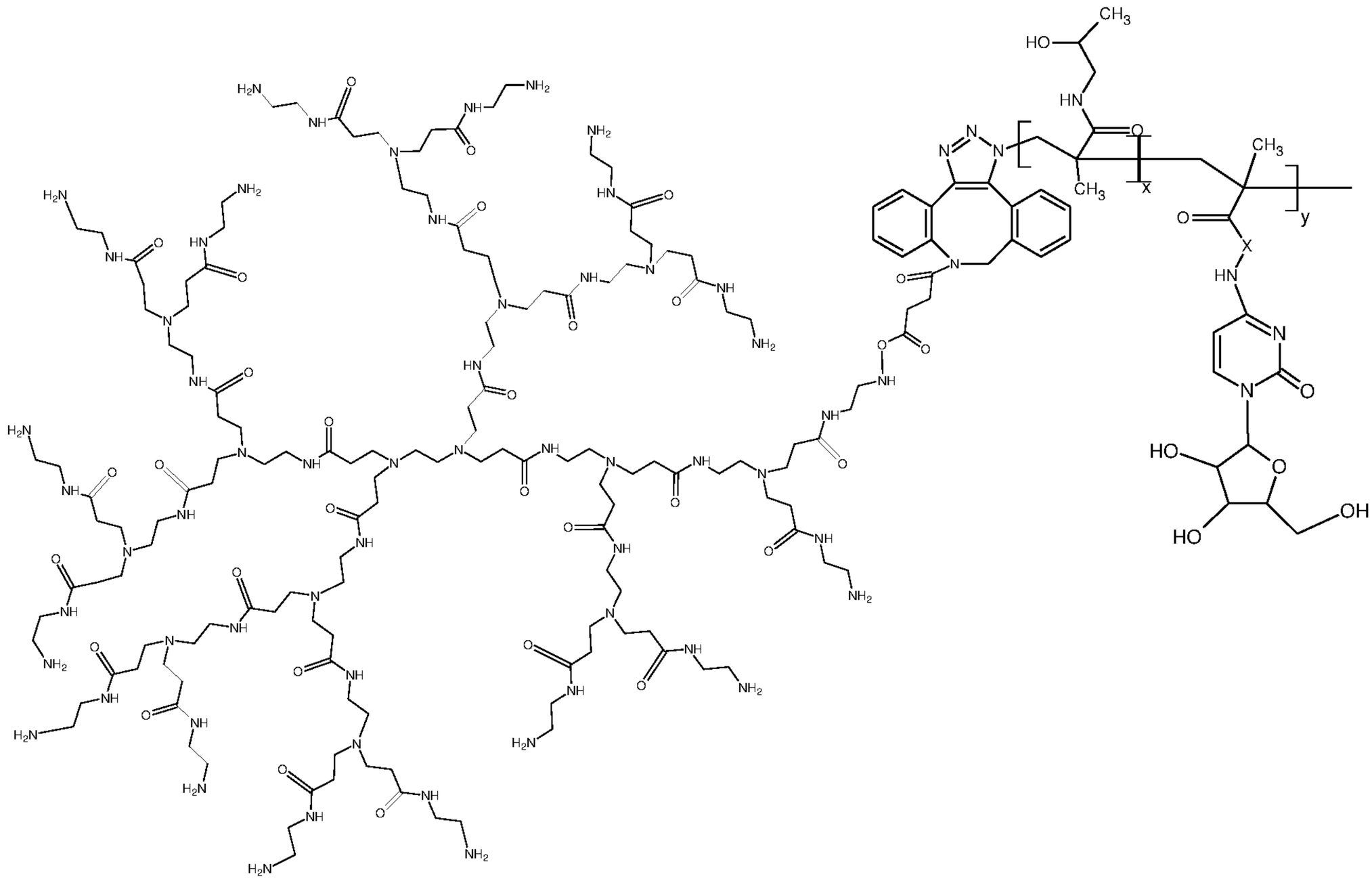
Example 8: Synthesis of star conjugate

The synthesis of the star copolymer proceeded in three steps. First, a reactive copolymer p(HPMA-*co*-MA-AH-TT)-N_3 was prepared using azide-CTA, *N*-(*3*-azidopropyl)-*4*-ethylsulphanylcarbothioylsulphanyl-*4*-methyl-pentanamide as transfer agent containing an azide group, in a manner similar to the reactive copolymer in Example 3. The copolymer of cytarabine was

prepared from this reactive polymeric precursor as in Example 4. In the second step, the second generation PAMAM dendrimer was modified by reacting its end amino groups with dibenzocyclooctyne N-hydroxysuccinimidyl ester (DBCO-NHS) as follows: 20 mg of PAMAM dendrimer G2 with amino groups (6.7 μmol of dendrimer, 0.1 mmol of amino groups) was dissolved in 1 mL of methanol and a solution of 5 mg of DBCO-NHS in 0.2 mL of methanol was added. After 1 h, unreacted DBCO was removed from the reaction mixture by gel chromatography (Sephadex LH20, methanol). The result was a PAMAM dendrimer containing end DBCO groups. The modified dendrimer PAMAM-DBCO in the last step reacted with p(HPMA-*co*-Ma-AH-araC)-N_3 in methanol for 2 h. The resulting star polymeric conjugate was precipitated into acetone and dried to constant weight. Characterisation of the resulting star polymeric conjugate: M_w = 250,000 g/mol, $Đ$ = 1.20, cytarabine content = 10.6 wt%. By changing the polymer / dendrimer core ratio and by changing the dendrimer generation, it is possible to control M_w of polymeric systems over a wide range. Similarly, a bis-MPA dendron or dendrimer containing alkyne or cycloalkyl groups can be used to prepare star conjugates.



Scheme 3: Synthesis and structure of star copolymers with the drug cytarabine

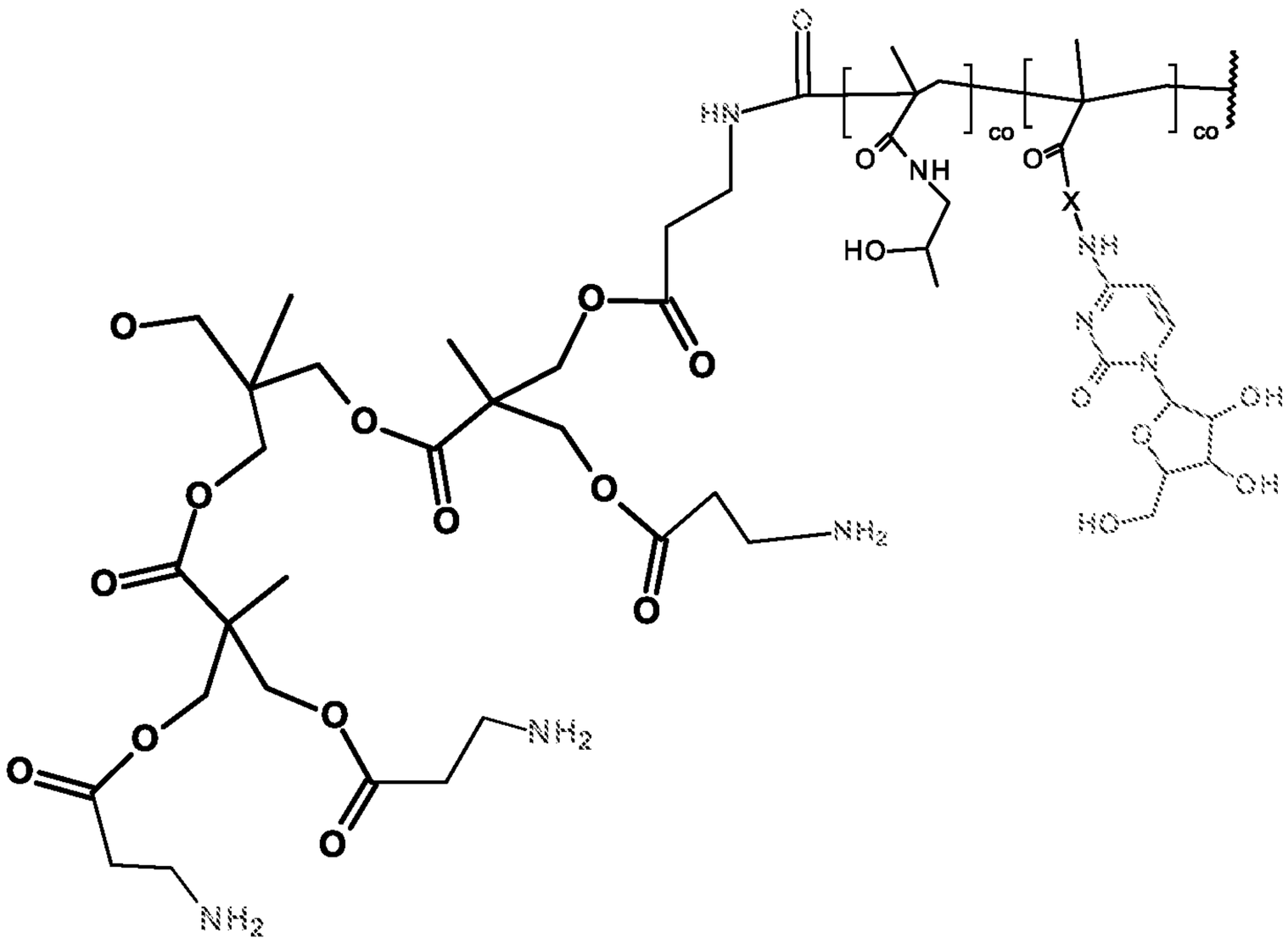


Scheme 4: Example of the structure of a second generation PAMAM dendrimer with a linear copolymer containing cytarabine linked via DBCO and a triazole bridge. The maximum number of chains of the linear copolymer that can be attached to the dendrimer is equal to the number of end groups of the dendrimer (in this case 16 end amino groups).

Example 9: Synthesis of a star polymeric conjugate with an amide linker

The synthesis of this star conjugate proceeded in two steps. First, the reactive copolymer p(HPMA-*co*-Ma-AH-araC)-TT was prepared using the transfer agent TT-CTA, [1-cyano-1-methyl-4-oxo-4- (2-thioxothiazolidin-3-yl)butyl]benzenecarbodithioate containing a TT group in a manner similar to the copolymer in Example 2. In the second step, the amino group-containing bis-MPA dendrimer was reacted with p(HPMA-*co*-Ma-AH-araC)-TT in methanol for 2 h. The resulting star polymeric conjugate was precipitated into acetone and dried to constant weight. Characterisation of the resulting star polymeric conjugate: M_w = 220,000 g/mol, $Đ$ = 1.18, cytarabine content = 10.1 wt%. By changing the polymer / dendrimer core ratio and by changing the dendrimer generation, it is possible to control

M_w of polymeric systems over a wide range. Similarly, bis-MPA dendron or PAMAM dendrimer with amino groups can also be used to prepare star conjugates.



Scheme 5: Example of the structure of a second generation bis-MPA dendron with a linear copolymer containing cytarabine linked via an amide bond. The maximum number of linear copolymer chains that can be attached to the dendron is equal to the number of end groups of the dendron (in this case 4 end amino groups).

Example 10: Release of cytarabine from polymeric conjugates

The amount of cytarabine released from polymeric conjugates with cytarabine linked via an amide bond to the polymer side chain after their incubation in phosphate buffer pH 7.4 (0.1 M phosphate buffer containing 0.05 M NaCl) modelling the bloodstream environment and phosphate buffer pH 5.0 modelling the intracellular environment was determined using the HPLC system (Shimadzu) equipped with the Chromolith Performance RP-18e reverse phase column (100 × 4.6 mm) and Shimadzu SPD-10AVvp UV-VIS detector (230 nm); eluent water-acetonitrile with a gradient of 0 to 100 vol.% of acetonitrile, flow rate 0.5 $mL \cdot min^{-1}$.

After incubation of the conjugates (0.5 mg/mL concentration) in a physiological environment modelling the bloodstream, pH 7.4, the polymeric conjugates were found to release small amounts of

drug, up to 7 % of the total content in 24 h (Fig. 1). In a slightly acidic environment at 37 °C (phosphate buffer, pH 5.0), cytarabine is released at a similar rate as in a pH 7.4 environment. The linker structure has a major effect on the rate of cytarabine release from the polymeric conjugate at pH 7.4. While in the case of GFLG and β-Ala linkers described in the literature about 20 and 10 % respectively of the total bound cytarabine is released in 24 hours, the rate of linkers described in the present invention is more than 3 times lower. It will be appreciated that all polymeric conjugates of the present invention have higher hydrolytic stability compared to prior art conjugates.

Thus, the polymeric conjugates of cytarabine described herein allow for significantly longer circulation of cytarabine in an inactive form and at the same time significantly protect the transported cytarabine against its degradation in the body. The polymeric conjugates have clearly shown high hydrolytic stability in the bloodstream environment, which prevents the release and subsequent deamination of transported cytarabine in the circulation, but the drug can accumulate in the form of polymeric conjugate in lymphoma, where the gradually released araC exhibits its anti-tumour activity.

The release of cytarabine from polymeric conjugates with cytarabine bound by a combination of amide and hydrazone bonds was similarly performed (Fig. 2). These polymeric systems were shown to have a higher stability at pH 7.4 than those described in the literature, so less drug is released than is in the case of GFLG and beta-Ala linkers. In addition, it has been found that when the pH drops to the values found in solid tumours and lymphomas, there is a significantly accelerated release, which should cause high anti-lymphoma activity.

Example 11: Demonstration of in vivo biological activity of linear polymeric conjugates of cytarabine in mice inoculated with mantle cell lymphoma (MCL)

A mantle cell lymphoma model derived from a patient with relapsed disease (VFN-M1 model) was used to demonstrate the activity of cytarabine conjugates *in vivo*. The immunodeficient mice NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (females) were xenotransplanted with $1x10^6$ VFN-M1 cells subcutaneously on day 0. The drug was administered once intravenously (i.v.) at a time when the tumours were well developed, palpable, and approximately 300 mm^3 in size. The tumour sizes in three perpendicular dimensions, the body weight of the mouse, overall health of the mouse and survival were monitored throughout the experiment. The effect of conjugate p(HPMA-*co*-MA-AH-araC) was compared with the effect of free cytarabine, the polymeric conjugate p(HPMA-*co*-MA-GFLG-araC) described in the literature and with the untreated control (Fig. 3). The polymeric drug conjugates were

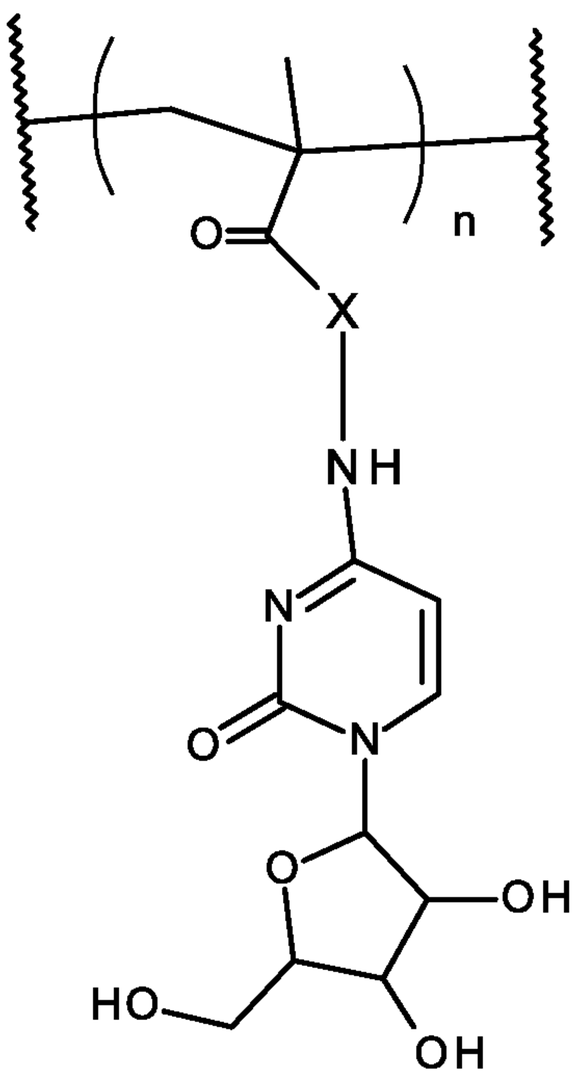
dissolved in PBS. The volume of each single dose of the drug was 0.2 mL. The following doses of drugs were applied: free cytarabine 4 mg/mouse, polymeric conjugate p(HPMA-*co*-MA-GFLG-araC) at a dose of 4 mg of araC/mouse equivalent and p(HPMA-*co*-MA-AH-araC) at a dose of 4 and 2 mg of araC/mouse equivalent.

Conclusion: The conjugate with the AH linker (4 and 2 mg of araC/mouse) has a significantly greater therapeutic effect in the treatment of MCL than the previously published conjugate with the GFLG linker (4 mg of araC/mouse) and than the free cytarabine alone. The higher hydrolytic stability of cytarabine during circulation in the bloodstream prolongs the circulation time and thus the amount of accumulated cytarabine in the lymphatic tissue. Thus, increased hydrolytic stability is clearly an advantage in the therapeutic effectiveness of these polymeric conjugates.

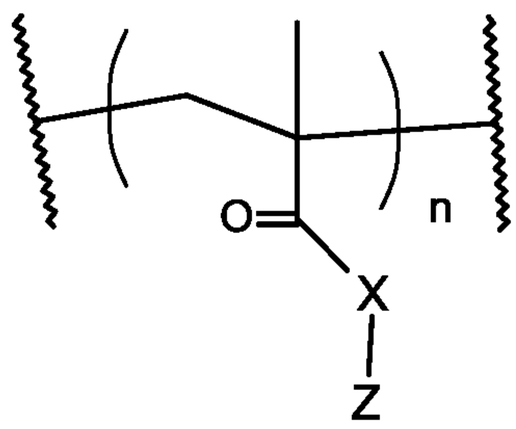
CLAIMS

1. A polymeric conjugate comprising from 80 to 95 mol% of monomeric units of HPMA and from 5 to 20 mol% of monomeric units of the general formula (I) and optionally of the general formula (II), based on the sum of monomeric units of HPMA, (I) and (II),

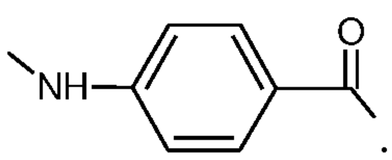
I



II



wherein

X is a covalent bond or an aminoacyl attached via an amide bond to the carbonyl group of the monomeric unit (I) and, optionally, to the carbonyl of the monomeric unit (II), and selected from the group consisting of $–NH–(CH_2)_n–C(=O)–$, $–NH–(CH_2)_q(C(=O)–NH–(CH_2)_r)_p–C(=O)–$, 

; $–NH–(CH_2)_n–C(=O)–NH–N=C(A)–(CH_2)_q–L–C(=O)–$;

wherein *n* is an integer from 1 to 6;

wherein *p*, *q* and *r* are independently selected from 1, 2 and 3;;

wherein *n* is an integer from 1 to 6; *q* is selected from 1, 2 and 3;

A is selected from the group consisting of methyl, isopropyl, cyclohexyl and pyridyl;

L is a covalent bond or 1,4-phenylene;

wherein –CH_2– of group X may be further substituted with one or more of the same or different natural amino acid side chains;

and/or the phenylene of group X may be further substituted with one or more of the same or different natural amino acid side chains;

preferably the natural amino acid chains are methyl, isopropyl, isobutyl, –$CH(CH_3)(CH_2CH_3)$, –CH_2OH, –$CH(OH)(CH_3)$, –CH_2–$(C_6H_4)OH$, –$(CH_2)_2$–S–CH_3, –CH_2SH, –$(CH_2)_4$–NH_2, –CH_2COOH, –$CH_2C(O)NH_2$, –$(CH_2)_2COOH$, –$(CH_2)_2C(O)NH_2$, –$(CH2)_3NH$–$C(=NH)(NH_2)$, benzyl;

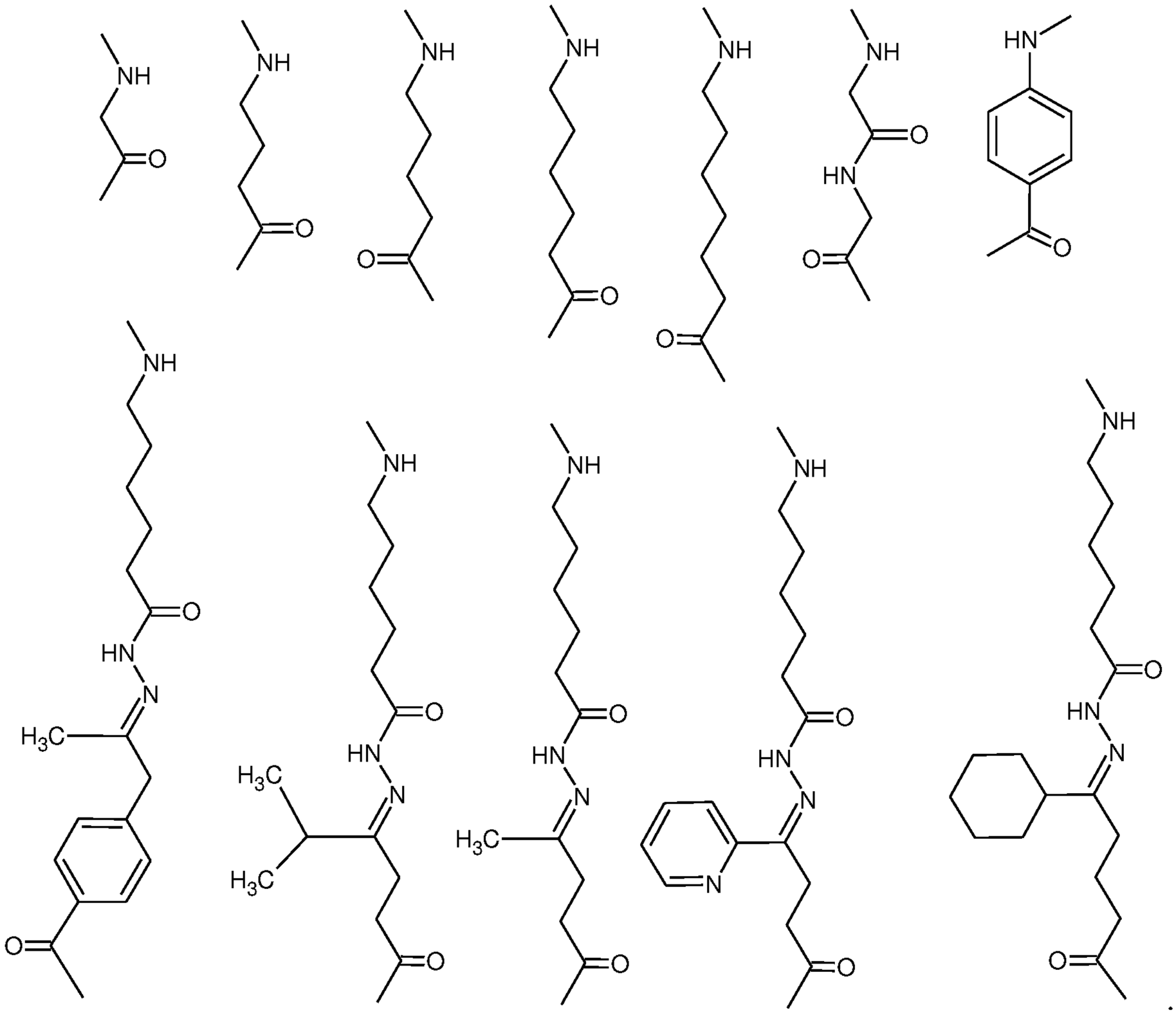
with the proviso that X is not –NH–$(CH_2)_2$–C(=O)– and that X is not –NH–CH_2–C(=O)–NH–CH(CH_2–Ph)–C(=O)–NH–CH(CH_2–iPr)–C(=O)–NH–CH_2–C(=O)–;

and Z is selected from the group comprising thiazoline-2-thion (TT); (4-nitrophenyl)oxy group; (2,3,4,5,6-pentafluorophenyl)oxy group; (succinimidyl)oxy group; hydrazide, –OH; -NH-CH_2-C(OH)-CH_3; –COOH; –C(=O)–NH–$(CH_2)_a$–$CH_2(OH)$; –C(=O)–NH–$(CH_2)_b$–CH(OH)–CH_3; –C(=O)–NH–$(CH_2)_b$–CH(OH)–$(CH_2)_c$–CH_3; wherein *a* is an integer from 0 to 4, *b* is an integer from 0 to 3 and *c* is an integer from 1 to 4;

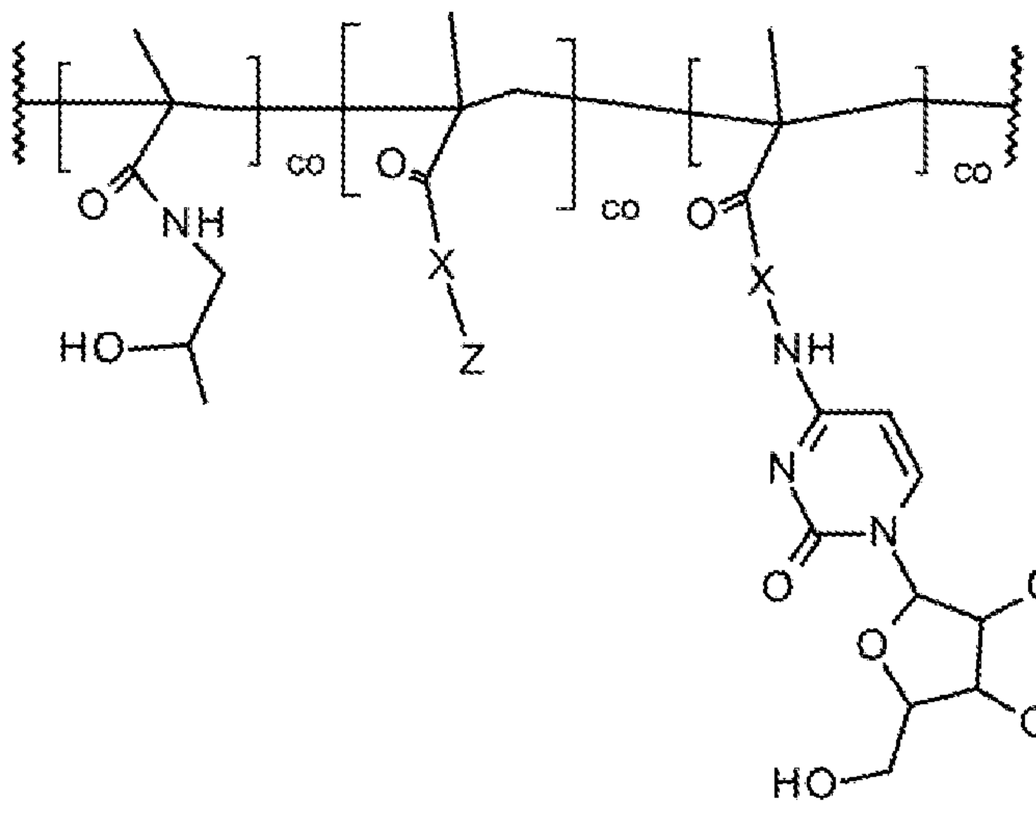
wherein in total the monomeric units of the general formula (I) and of the general formula (II) are at most 20 mol%, and the total amount of the monomeric units of the general formula (I) is at least 5 mol%, based on the sum of monomeric units of the HPMA, (I) and (II);

and wherein the molecular weight M_n of the polymeric conjugate is in the range of from 4,000 to 1,000,000 g/mol.

2. The polymeric conjugate according to claim 1, wherein X is a covalent bond or X is selected from the group consisting of:



3. The polymeric conjugate according to claim 1 or 2, which is a linear statistical copolymer of general formula (III),

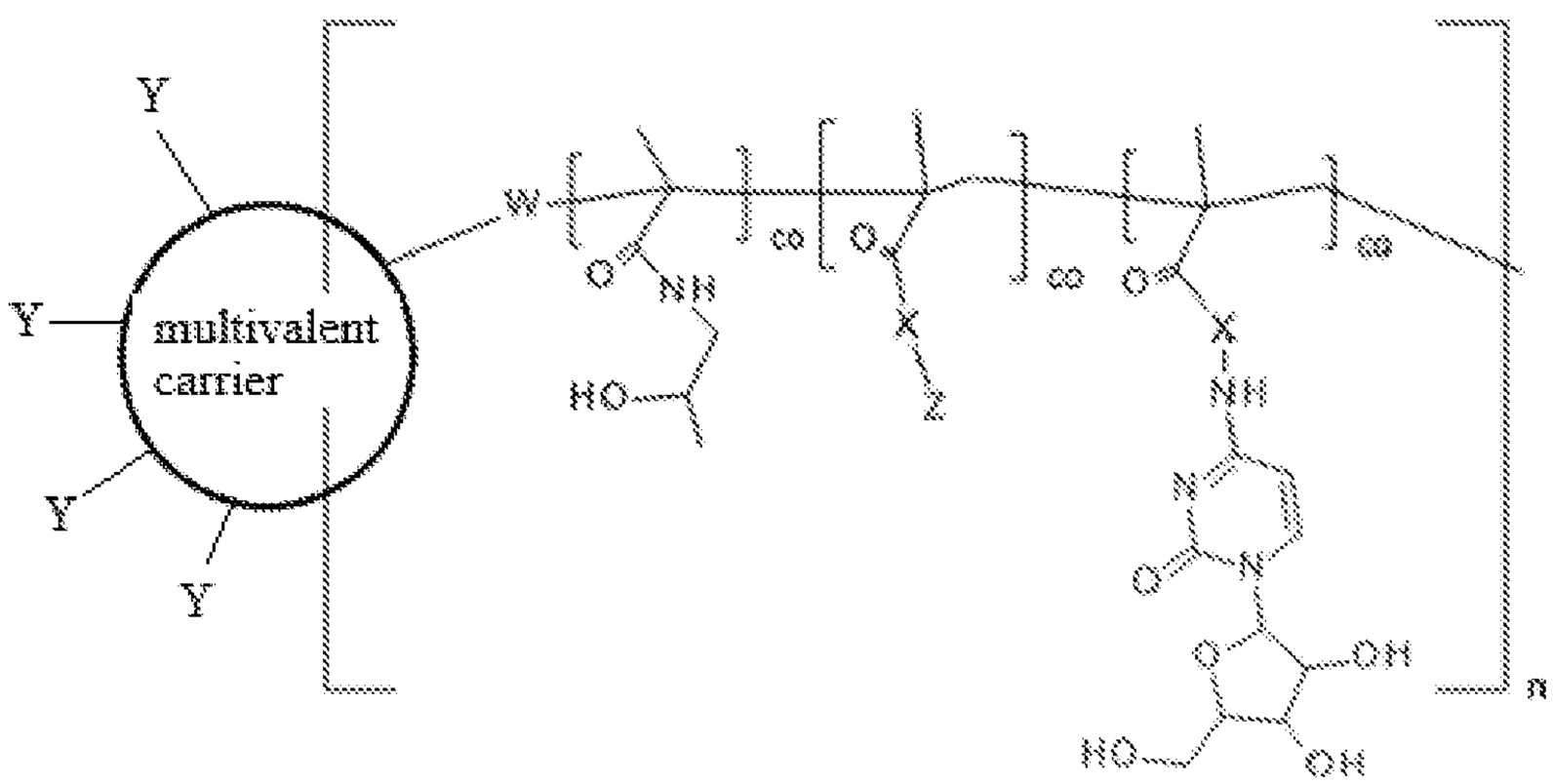


(III),

wherein X and Z are as defined in claim 1,

and wherein the molar mass of the linear statistical copolymer is in the range of from 4,000 to 100,000 g/mol, preferably from 40,000 to 70,000 g/mol.

4. The polymeric conjugate according to claim 1 or 2, which is a star copolymer of general formula (IV),



(IV),

wherein

n is an integer in the range of from 1 to 48;

the multivalent carrier is selected from the group consisting of a second or third generation poly(amidoamine) dendrimer or 2,2-bis(hydroxymethyl)propionic acid based second, third or fourth generation dendrimer or dendron;

X and Z are as defined in claim 1;

Y is selected from the group consisting of a primary amino group; alkyne group of 3 to 6 carbons; and a cyclooctyne group, which may be optionally further independently substituted with one or more groups selected from an alkyl group of 1 to 6 carbons and aryl of 6 carbons;

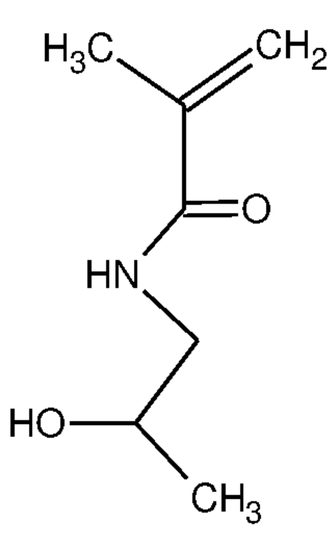
W is an amide bond or a triazole linker;

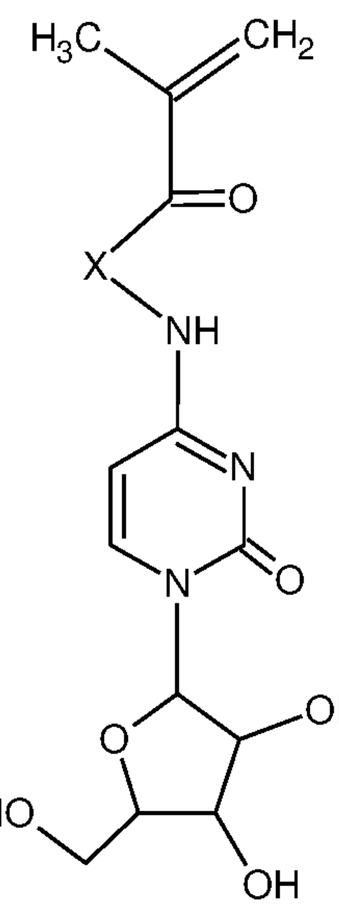
wherein the molar mass of the copolymer of general formula (IV) is in the range of from 60,000 to 1000,000 g/mol, preferably from 100,000 to 400,000 g/mol.

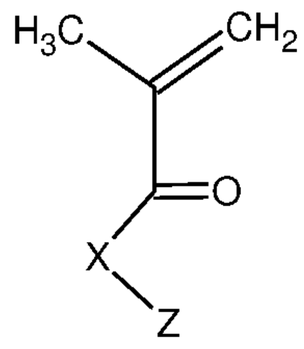
5. The polymeric conjugate according to claim 4, wherein the polymeric carrier has its molar mass less than 50,000 g/mol.

6. A method for the preparation of the linear statistical polymeric conjugate of general formula (III) according to claim 3, **characterized in that** it comprises the following steps:

(i) providing an HPMA monomer and a monomer of general formula (V) and/or (VI)







HPMA (V) (VI);

wherein

X is as defined in claim 1;

Z is selected from the group comprising thiazoline-2-thione (TT); (4-nitrophenyl)oxy group; (2,3,4,5,6-pentafluorophenyl)oxy group; (succinimidyl)oxy group; Boc–NH–NH–; –OH; -NH-CH_2-C(OH)-CH_3; –COOH; –C(=O)–NH–$(CH_2)_a$–CH_2(OH); –C(=O)–NH–$(CH_2)_b$–CH(OH)–CH_3; –C(=O)–NH–$(CH_2)_b$–CH(OH)–$(CH_2)_c$–CH_3; wherein *a* is an integer from 0 to 4, *b* is an integer from 0 to 3 and *c* is an integer from 1 to 4;

(ii) the step of radical polymerisation of HPMA with monomers of the general formula (V) and/or (VI);

(iii) in case the step (ii) is the polymerisation of HPMA with the monomer of general formula (VI), the step of attaching cytarabine to the Z group of the polymer of step (ii);

(iv) removal of the reactive end groups.

7. The method according to claim 6, **characterized in that**

- step (ii) takes place at a temperature in the range of from 30 to 100 °C, in a solvent preferably selected from the group comprising water, aqueous buffers, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol, dioxane, *tert*-butyl alcohol or mixtures thereof; initiated by an initiator, preferably selected from the group comprising, in particular, azo initiators 2,2′-azobis(2-methylpropionitrile), 4,4′-azobis(4-cyanopentanoic acid), 2,2'-azobis(4-methoxy-2,4-dimethylpentanenitrile);

optionally in the presence of a transfer agent, preferably selected from the group comprising 2-cyano-2-propylbenzodithioate, 4-cyano-4-(thiobenzoylthio)pentanoic acid, 2-cyano-2-propyldodecyl-trithiocarbonate, 2-cyano-2-propylethyltrithiocarbonate and 4-cyano-4-[(dodecylsulphanylthio-carbonyl)sulphanyl] pentanoic acid;

- step (iii) takes place at a temperature between 20 and 100 °C, in a solvent preferably selected from the group comprising pyridine, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol or mixtures thereof;

- in step (iv), the end groups of the linear polymer from step (ii) or (iii) react with an excess of azo initiator selected from the group of initiators described in step (ii), at a temperature in the range of from 50 to 100 °C, in a solvent preferably selected from the group comprising dimethylsulphoxide, dimethylacetamide and dimethylformamide, to form the polymer of general formula (III).

8. A method for the preparation of the star copolymer of general formula (IV) according to claim 4 or 5, **characterized in that** it comprises the following steps:

(i) providing an HPMA monomer and a monomer of general formula (V) and/or (VI);

(ii) radical copolymerisation of HPMA with monomers of general formula (V) and/or (VI) at a temperature in the range of from 30 to 100 °C; in a solvent preferably selected from the group comprising water, aqueous buffers, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol, dioxane, *tert*-butyl alcohol or mixtures thereof; initiated by an initiator, preferably selected from the group comprising, in particular, azo initiators 2,2′-azobis(2-methylpropionitrile), 4,4′-azobis(4-cyanopentanoic acid), 2,2'-azobis (4-methoxy-2,4-dimethylpentanenitrile); in the presence of a transfer agent selected from the group comprising [4-(3-azidopropylamino)-1-cyano-1-methyl-4-oxo-butyl]benzenecarbodithioate, N-(3-azidopropyl)-4-cyano-4-ethylsulphanyl-carbothioylsulphanyl-pentanamide, [1-cyano-1-methyl-4-oxo-4-(2-thioxothiazolidin-3-yl)butyl] benzenecarbodithioate and 2-ethylsulphanylcarbothioyl-sulphanyl-2-methyl-5-oxo-5-(2-thioxo-thiazolidin-3-yl)pentanenitrile;

wherein the molar mass M_n of the linear copolymers thus prepared is in the range of from 4,000 to 100,000 g/mol, preferably from 25,000 to 50,000 g/mol; wherein the reactive end groups, azide and TT, introduced by the transfer agent, are further used in step (iii);

(iii) providing a multivalent carrier for grafting the semitelechelic copolymer of step (ii), wherein the multivalent carrier is selected from the group consisting of a second or third generation poly (amidoamine) dendrimer and 2,2-bis(hydroxymethyl) propionic acid (bis-MPA) based second, third or fourth generation dendrimer or dendron, terminated by Y groups selected from primary amino groups, alkyne groups of 3 to 6 carbons, and cyclooctyne groups which may be optionally further independently substituted with one or more groups selected from an alkyl group of 1 to 6 carbons and aryl of 6 carbons;

(iv) step of grafting the reactive functional groups of the linear semitelechelic copolymer of general formula (III) prepared in step (ii) to the Y groups of the multivalent carrier from step (iii) to form the star polymeric conjugate of general formula (IV);

(v) optionally, if the resulting star polymeric conjugate of step (iv) contains monomeric units of general formula (II), the step of attaching cytarabine to the Z group of the polymer of step (iv) at a temperature between 20 and 100 °C, in a solvent preferably selected from the group comprising

pyridine, dimethylsulphoxide, dimethylacetamide, dimethylformamide, methanol, ethanol or mixtures thereof.

9. A pharmaceutical composition, **characterized in that** it comprises as active ingredient the polymeric conjugate according to any one of the preceding claims 1 to 5; wherein the pharmaceutical composition further comprises at least one pharmaceutically acceptable excipient, selected from the group comprising anti-adhesives, binders, coating agents, colourants, swelling agents, flavouring agents, lubricants, preservatives, sweeteners, sorbents, solvents, buffers, ionising additives, antioxidants, anti-microbial additives.

10. The polymeric conjugate according to any one of the preceding claims 1 to 5 and/or the pharmaceutical composition according to claim 9 for use as a medicament, preferably for use in the treatment of malignancies, more preferably for the treatment of solid tumours and/or haematological tumours, most preferably for the treatment of leukaemias and non-Hodgkin's lymphomas.

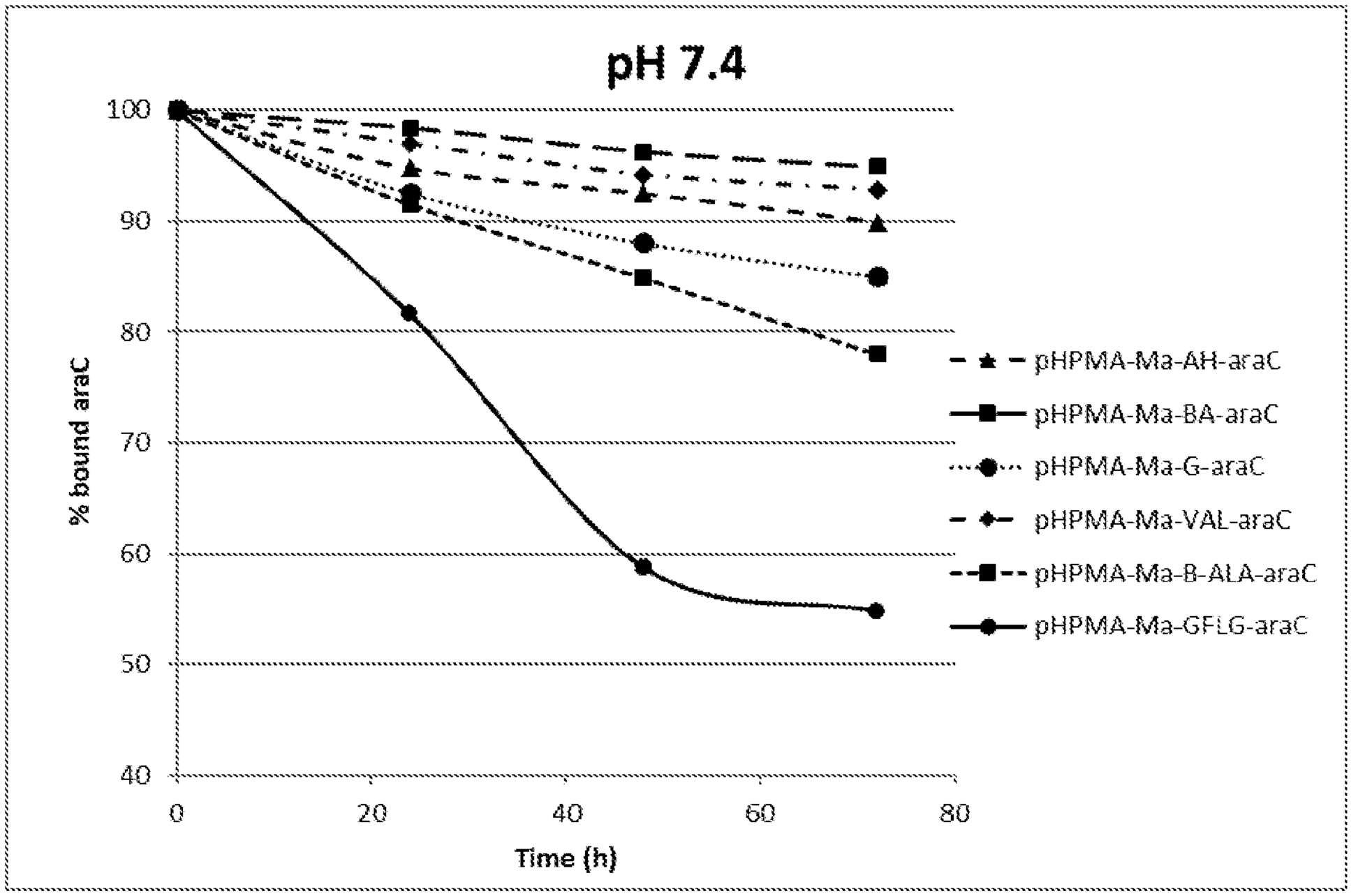

pH 7.4
100
90
80
70
60
50
40
% bound araC
0
20
40
60
80
Time (h)
pHPMA-Ma-AH-araC
pHPMA-Ma-BA-araC
pHPMA-Ma-G-araC
pHPMA-Ma-VAL-araC
pHPMA-Ma-B-ALA-araC
pHPMA-Ma-GFLG-araC

Fig. 1

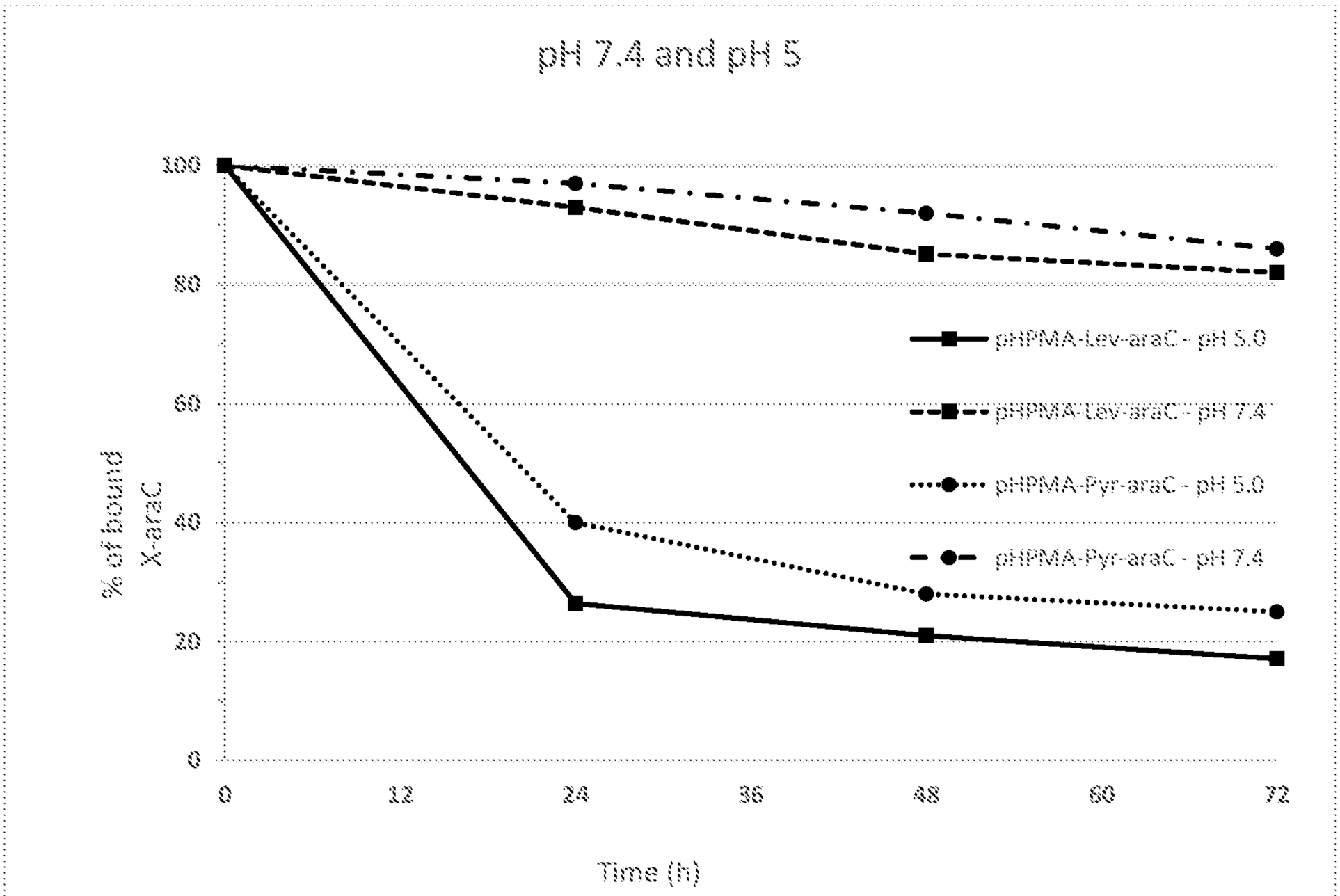

pH 7.4 and pH 5
100
80
60
40
20
0
% of bound X-araC
0
12
24
36
48
60
72
Time (h)
pHPMA-Lev-araC - pH 5.0
pHPMA-Lev-araC - pH 7.4
pHPMA-Pyr-araC - pH 5.0
pHPMA-Pyr-araC - pH 7.4

Fig. 2

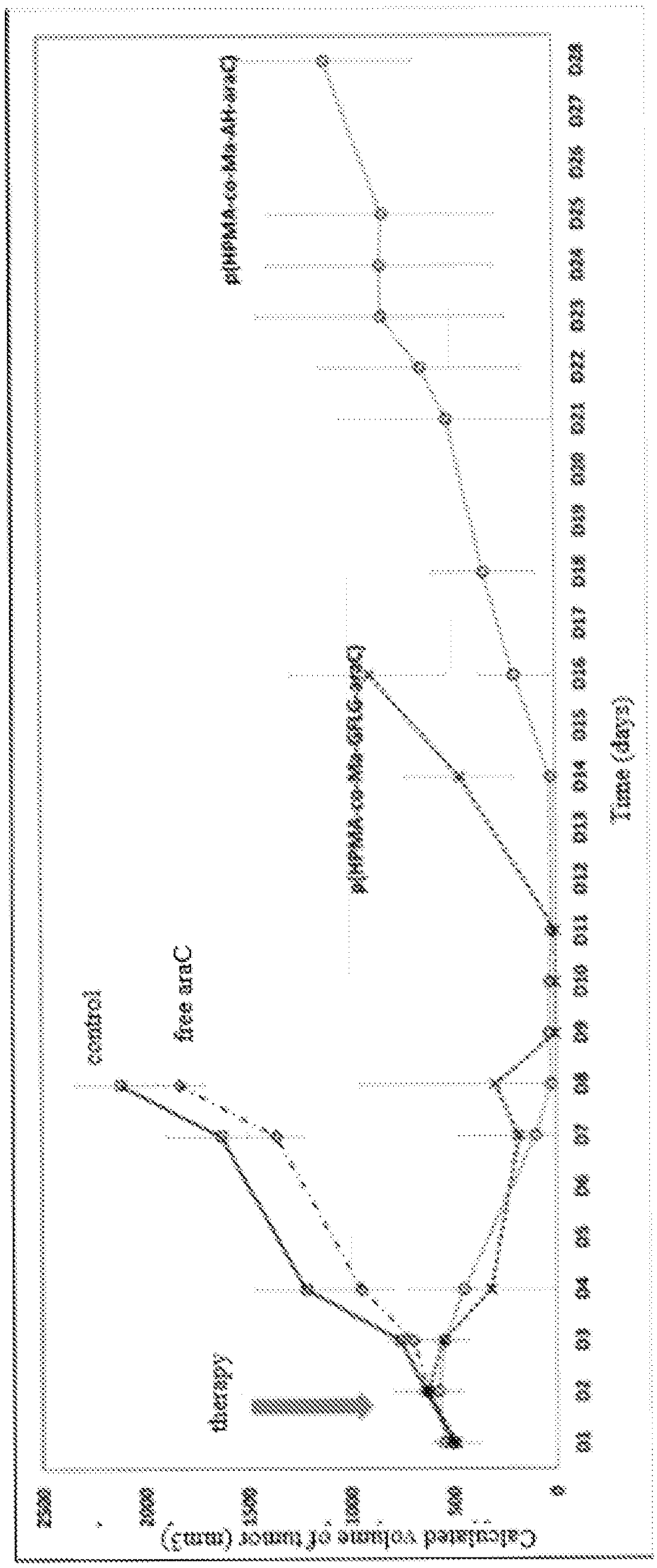

calculated volume of tumor (mm3)
therapy
control
free araC
time (days)

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2020/050097

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K47/58
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2017/019559 A1 (UNIV UTAH RES FOUND [US]) 2 February 2017 (2017-02-02)	1,3-10
A	whole documents, in particular [0124]	2

Y	R. POLA ET AL: "The pH-Dependent and Enzymatic Release of Cytarabine From Hydrophilic Polymer Conjugates", PHYSIOLOGICAL RESEARCH., vol. 65, no. 2, 17 September 2016 (2016-09-17), pages S225-S232, XP055768399, CZ ISSN: 0862-8408, DOI: 10.33549/physiolres.933424	1,3-10
A	whole document, in particular figure 1	2

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 March 2021	24/03/2021
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	**Authorized officer** Schleifenbaum, A

1

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INTERNATIONAL SEARCH REPORT

International application No
PCT/CZ2020/050097

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	ULBRICH K ET AL: "HPMA copolymers with pH-controlled release of doxorubicin - In vitro cytotoxicity and in vivo antitumor activity", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 87, no. 1-3, 21 February 2003 (2003-02-21), pages 33-47, XP004412737, ISSN: 0168-3659, DOI: 10.1016/S0168-3659(02)00348-6 figure 1	1,3-10
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Y	WO 2008/034391 A1 (ZENTIVA AS [CZ]; ETRYCH TOMAS [CZ] ET AL.) 27 March 2008 (2008-03-27) claim 4	1,3-10
Y	YANG JIYUAN ET AL: "The light at the end of the tunnel-second generation HPMA conjugates for cancer treatment", CURRENT OPINION IN COLLOID & INTERFACE SCIENCE, vol. 31, 28 July 2017 (2017-07-28), pages 30-42, XP085232211, ISSN: 1359-0294, DOI: 10.1016/J.COCIS.2017.07.003 figure 2	1,3-10

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/CZ2020/050097

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