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Synthesis of specific nanoparticles for targeting and imaging tumor angiogenesis using electron-beam irradiation

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Abstract

Angiogenesis plays a critical role in both growth and metastasis of tumors. Vascular endothelial growth factor (VEGF) is an endogenous mediator of tumor angiogenesis. Blocking associations of the VEGF with its corresponding receptors (KDR) have become critical for anti-tumor angiogenesis therapy. A cyclo-peptide (CBO-P11), derived from VEGF, able to inhibit angiogenesis was synthesized in our laboratory. We have prepared biocompatible poly(vinylidene fluoride) (PVDF) nanoparticles in order to obtain long circulating drug delivery systems. These particles were characterized and they were found monodisperse with a mean radius of 60 nm. Electron-beam (EB) irradiation was used to activate PVDF nanoparticles. From electron paramagnetic resonance (EPR) measurements, we studied the radical stability in order to optimize the radiografting of acrylic acid (AA). Further functionalization of PVDF-g-PAA nanoparticles with the cyclo-peptide *via* a spacer arm was also possible by performing coupling reactions. High resolution magic angle spinning nuclear magnetic resonance (HRMAS NMR) and MALDI mass spectrometry allowed us to follow each chemical step of this peptide immobilization. 7727-21-1

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1. Introduction

Angiogenesis, the formation of new capillary blood vessels from pre-existing vasculature, plays an essential role in normal processes, such as embryogenesis, wound healing and in pathological processes like tumor growth (Heljasvaara et al, 2005). Inhibition of angiogenesis represents then a promising strategy to block tumor growth and invasion. A number of endogenous angiogenic regulators such as VEGF, fibroblast growth factor (FGF) and angiopoietins have been identified (Zilberberg et al, 2003). VEGF and its receptors (VEGFR-1 and VEGFR-2 which are tyrosine kinase activity) are frequently up-regulated in a number of clinically important human diseases, including cancer, making them an attractive target for therapies (Miao et al, 2006). Different strategies have been designed to inhibit VEGF function by blocking its interaction with its receptor (Keedy et al, 2007; Zilberberg et al, 2003). A 17-amino acid cyclo-peptide was previously described as a vascular growth inhibitor (CBO-P11) (Zilberberg et al, 2003). This molecule encompasses residues 79-93 of VEGF which are involved in the interaction with its receptor and shows a micromolar affinity for VEGFR-2.

In order to improve circulation time of the peptide, nanoparticles may be used for the transportation of the drug to the target tissue. Nanoparticles most commonly refer to solid colloidal particles made of macromolecular material ranging from 1 to 1000 nm. They can be used as drug carriers, either by dissolving, entrapping, encapsulating or attaching the active substance. Various types of carriers have been developed such as polymeric micelles, polymer-based nanoparticles and liposomes (Van Butsele et al, 2007). Therapeutically used polymeric nanoparticles are composed of biodegradable hydrophobic polymers protected by an amphiphilic block copolymer that stabilizes their dispersion in aqueous media (Sung et al, 2007; Van Butsele et al, 2007). With regard to the hydrophobic core, we are interested in a fluorinated polymer, poly(vinylidene fluoride) (PVDF). This latter is a semicrystalline

thermoplastic, biocompatible polymer, remarkable for its physical and chemical resistance. In addition to numerous industrial applications, PVDF shows new interests in biotechnology (microporous and ultrafiltration membranes) and in biomedical activity (vascular sutures, regenerated templates) (Braga et al, 2007; Chen et al, 2006a; Chen et al, 2006b; Marchand-Brynaert et al, 1997). However, no literature to our knowledge reports on the use of PVDF nanoparticles as a carrier for drug delivery.

The aim of the present paper is to immobilize of a bioactive peptide such CBO-P11 onto PVDF nanoparticles. PVDF presents a high hydrophobicity. Therefore, to improve its hydrophilicity, the nanoparticles were coated with poly(acrylic acid) (PAA) using electron-beam irradiation to obtain a grafted copolymer, PVDF-g-PAA (Betz et al, 2003; Clochard et al, 2004). PAA carboxylic acid functions allow the coupling of a spacer arm to occur on nanoparticles and CBO-P11 was covalently linked to the spacer arm by click chemistry reaction (**Scheme 1**). Every step has been characterized by HRMAS NMR or MALDI mass spectrometry.

2. Experimental part

Synthesis of PVDF nanoparticles: The synthesis has taken place at PiezoTech SA. Poly(vinylidene fluoride) nanoparticles were prepared in a reactor under pressure by nanoemulsion polymerization of corresponding monomer (VF_2) as described in a previous paper (Kappler et al, 2004). The monomer is emulsified in a continuous phase of water using potassium persulfate as initiator and perfluorooctanoic acid as ionic surfactant. These perfluorinated surfactants promotes micellization at low concentration (Moody et al, 2000). To stabilize the emulsion, paraffin wax was used as dispersing agent.

Irradiation: Samples of lyophilized PVDF nanoparticles were put inside sealed glass tubes under vacuum. Irradiations were performed at room temperature using a 10 MeV Pulsed Electron Beam industrial accelerator at Ionisos (Chaumesnil, France). Irradiation doses lies in range of 25 to 200 kGy.

Grafting: The irradiated powder of lyophilized PVDF nanoparticles was dispersed into a grafting aqueous solution of AA. Grafting experiments were performed at 60°C for 1 h. At this temperature, two chemical reactions occur: i) thermal homopolymerization of AA, and ii) grafting reaction on the nanoparticles. Therefore, to avoid homopolymerization, we have added 0.25 wt% of Mohr's salt (**Scheme 1**). The nanoparticles were purified by centrifugation and dialysis. Finally, they were freeze-dried to obtain a white powder of copolymer (PVDF-g-PAA **[1]**).

PVDF-g-PAA nanoparticles decoration: mTEG was coupled to **[1]** *via* an amide bond to the carboxylic acid function of PAA using ethyl-3(3dimethylaminopropyl)carbodiimide (EDC) in an aqueous solution at room temperature for 24 h (**Scheme 1**). The nanoparticles were purified by centrifugation and dialysis. Finally, they were freeze-dried to obtain a brown powder (PVDF-g-PAA-mTEG **[2]**). CBO-P11 or cyclo-VEGI (*D*-FPQIMRIKPHQGQHIGE) was synthesized by Fmoc/t-Bu batch solid-phase synthesis (Goncalves et al, 2005). After cleavage from the resin and before deprotection of the peptide, propargylamine was coupled to the glutamic acid unit. Finally, the peptide was deprotected and purified by reversed-phase HPLC. Then, the purified peptide **[3]** was coupled to **[2]** by click chemistry using copper sulfate and sodium ascorbate at 40°C for 3 days. The nanoparticles were purified by centrifugation with an EDTA solution in order to remove copper. The supernatant was collected, purified by reverse-phase HPLC in order to quantify unreacted peptide and to

determine indirectly the grafting yield. Then, the nanoparticles were dialysed and freeze-dried to obtain PVDF-g-PAA-mTEG-P11 **[4]** as a white powder.

Field Emission Scanning Electron Microscope (FESEM): A Hitachi S-4800 field emission scanning electron microscope equipped with a tip made of Zr monocrystal allowed us to take pictures of the fragile PVDF nanoparticles without metallization. Accelerating voltage: 1kV. Tip current : 10 µA; Probe current: Normal; UltraHigh Resolution Mode; Condenser lens : 5; Focus depth : 1; Objective lens diaphragm : 2 ; Working distance : 2 mm.

Dynamic light scattering (DLS): A Zetasizer Nano-ZS dynamic light scattering (Malvern instrument 3000HSA) was used for nanoparticles characterization.

Small Angle Neutron Scattering (SANS): Measurements were performed on PACE spectrometer at LLB (CEA-Saclay). Nanoparticle latexes were diluted 10 times in D_2O. For each measurements, neutron scattered intensity was recorded as a function of scattering vector. Data treatment was performed following a previous paper (Brulet et al, 2007). Scattered intensity profile is accounted for using the form factor of a sphere.

Electron Paramagnetic Resonance (EPR): EPR spectra were recorded at the X band (9.4GHz) on a Brüker ER-200D EPR spectrometer.

3. Results and discussion

Characterization of PVDF nanoparticles

The particle size was determined by FESEM, by Zetasizer Nano-ZS dynamic light scattering (DLS) (Malvern instrument 3000HSA) and by small angle neutron scattering (SANS). Results obtained by these different characterization methods are similar. The radius of nanoparticles is around 60 nm with a polydispersity index of 0.002 determined by DLS,

indicating a monodisperse latex. A typical FESEM image is shown in **Fig. 1**, PVDF nanoparticles appear spherical and monodisperse.

Radical stability

Under vacuum, electron beam irradiation generates mainly alkyl radicals inside the PVDF polymer bulk. When opening the irradiation tube, the PVDF nanoparticles come in contact with air. Alkyl radicals combine immediately with oxygen to form peroxide radicals. The radical amount is proportional to the absorbed dose. If the irradiation dose is important, a yellowish colour appears corresponding to unsaturated bound creation. Figure 2 shows EPR spectra at different doses. The hyperfine band at 3520 Gauss observed at 200 kGy is due to polyenyl formation. The EPR spectrum corresponding to irradiation dose of 50 kGy of a sample opened for 3 months is similar to the one corresponding to irradiation dose of 25 kGy. Nanoparticles radical content is consequently far to be stable with time. This behaviour is different from what it has been already observed for PVDF film (Clochard et al, 2004). The high stability in films is assumed coming from radical trapping inside the polycrystalline part of the PVDF (Aymes-Chodur et al, 1999; Aymes-Chodur et al, 2001; Clochard et al, 2004). A DSC study (**Fig. 3**) allows us to determine the crystallinity rate of the nanoparticles at various doses. For a 100% crystalline PVDF, $\Delta H_{PVDF\ 100\%\ crystalline}$ is equal to -25 cal/g or -104,5 J/g. Nanoparticles enthalpies were found equal averagely to $\Delta H_{PVDFnanoparticle}$= -29,5 J/g whatever the irradiation dose and $\Delta H_{PVDFfilm}$= -38 J/g corresponding to 28 ± 2 % et 36 ± 2% crystallinity rates respectively. Results show a diminution of crystallite content for nanoparticles in comparison with films. Moreover, the major variation observed in DSC curves (**Fig.3**) is the shift in melting peak to lower temperature. Indeed, the melting temperature (Tm) is at 160°C for the nanoparticles and at 167°C for the films. It means that the crystallite size is much smaller in nanoparticles. Electron beam irradiation seems to not

affect the crystallinity rate of nanoparticles. Consequently, the radical decay with time in nanoparticles may be due to small crystallite content. The high specific area of nanoparticles may also participate to this radical consumption. Indeed, the available area for oxygen to combine with alkyl radicals is huge compared to a flat film and peroxide radicals are well-known to be less stable. Consequently, it is of great importance to radiograft quickly after irradiation.

Radiation Grafting of PVDF with acrylic acid.

Fig.4 shows proton NMR spectra of PVDF and PVDF-g-PAA **[1]** recorded in DMF-*d7*. In PVDF spectrum, the large triplet at 3.03 ppm corresponds to the signal of CH_2 repeat unit and the triplet at 2.5 corresponds to CH_3 signal of PVDF chain ends. The PVDF-g-PAA spectrum displays large peaks at 1.98 and 1.73 ppm corresponding to the CH_2 signal of PAA. CH of PAA gives rise to a signal at 2.5 ppm. From integrated signals, a quantitative approach allows us to evaluate the PAA/PVDF ratio. The resulting yield for PVDF-g-PAA is found equal to 56 mol%.

Covalent coupling of spacer arm.

A spacer arm onto nanoparticles brings mobility to the future immobilized peptide. We synthesized a modified tetraethylene glycol (noted mTEG) with an amine function at one end and an azide function at the other end. This linker was chosen because of its solubility and a previous study shows that this spacer arm did not affect the activity of CBO-P11 (Goncalves et al, 2005). The HRMAS NMR spectrum of **[2]** is shown in **Fig.4** and displays two peaks for CH_2 of the spacer arm ($C\mathbf{H_2}C\mathbf{H_2}O$ and $C\mathbf{H_2}NH$) at 3.63 and 3.67 ppm. On the other hand, the signal corresponding to $C\mathbf{H_2}N_3$ is masked by the water peak at 3.46 ppm. Integration of CH_2 (mTEG) signal allows us to evaluate the mTEG/PVDF ratio and to determine a 31 mol%

yield for PVDF-g-PAA-mTEG. It means that only 50% of the available carboxylic acids were covalently bound to spacer arm.

Peptide synthesis and coupling to nanoparticles by click chemistry

In order to attach the peptide CBO-P11 onto nanoparticles, a functionalization of the original peptide is needed. Propargylamine was coupled to the glutamic acid unit in order to have an alkyne function (**Scheme 1**). This later allows further anchoring *via* click chemistry reaction to the spacer arm without affecting other side chains of the peptide which are essential for the recognition with VEGFR-2 receptors. The peptide coupling was proved by MALDI mass spectrometry. The analysis of nanoparticles was performed in positive mode with Voyager STR mass spectrometer (Applied Biosystems). The signal at *m/z* 1998 Da corresponds to the CBO-P11 mass. It indicates the peptide grafting. From the supernatant-removed peptide, the grafting yield for PVDF-g-PAA-mTEG-P11 **[4]** was found equal to 5.5 mol%.

4. Conclusion

We have succeeded to synthesize PVDF nanoparticles by nanoemulsion polymerization and their functionalization with a peptide that presents an anti-angiogenic activity. Resulted nanoparticles present a radius of 60 nm. From FESEM images and light scattering measurements, we deduced that they were spherical and monodisperse. The alkyl radicals induced from electron beam irradiation combine immediately with the oxygen to form peroxide radicals. Because of a high specific area and small crystallite size, the radical decay with time is evidenced from EPR measurements. Despite this radical decay, electron beam irradiation allows us to graft PAA by radical polymerization onto freshly irradiated PVDF nanoparticles and then to immobilize CBO-P11 by click chemistry *via* a spacer arm.

Evidences of grafting were shown using HRMAS NMR and MALDI-TOF mass spectrometry. Nanoparticles functionalized with an angiogenesis-targeting agent are an attractive option for anti-tumor therapy.

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