Deposition of Nanoparticles in the Arterial Vessel by Porous Balloon Catheters: Localization by Confocal Laser Scanning Microscopy and Transmission Electron Microscopy

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Ulrich Westedt¹, Lucian Barbu-Tudoran², Andreas K. Schaper², Marc Kalinowski³, Heiko Alfke³, and Thomas Kissel¹

¹Department of Pharmaceutics and Biopharmacy, Philipps-University, D-35032 Marburg, Germany

²Materials Science Center, Philipps-University, D-35032 Marburg, Germany

³Department of Diagnostic Radiology, Philipps-University, D-35032 Marburg, Germany

ABSTRACT Restenosis remains the major limitation of percutaneous transluminal angioplasty (PTA) and stenting in the treatment of patients with atherosclerotic disease. Catheter-based local delivery of pharmacologic agents offers a potential therapeutic approach to reducing restenosis and minimizing undesirable systemic side effects. However, the intramural retention of liquid agents is low. Therefore, to achieve a sustained and regional release of the therapeutic agent it must be encapsulated in nanoparticle carrier systems. The purpose of this study was to investigate the size dependence of the penetration of nanoparticles after local delivery into the vessel wall of the aorta abdominalis of New Zealand white rabbits. Two milliliters of a 0.025% fluorescence-labeled polystyrene nanoparticle suspension with diameters ranging from 110 to 514 nm were infused at 2 atm and at constant PTA pressure of 8 atm into the aorta abdominalis. After the infused segments were removed, the location of nanoparticles was visualized using confocal laser scanning microscopy and transmission electron microscopy. The study demonstrates a size-dependent nanoparticle penetration into the intact vessel wall. While nanoparticles of about 100 and 200 nm were deposited in the inner regions of the vessel wall, 514-nm nanoparticles accumulated primarily at the luminal surface of the aorta. The observations confirm that size plays a critical role in the distribution of particles in the arterial vessel wall. It is additionally influenced by the formation of pressure-induced infusion channels, as well as by the existence of anatomic barriers, such as plaques, at the luminal surface of the aorta or the connective elastic tissue.

KEYWORDS: angioplasty, restenosis, local deposition, nanoparticles.

Correspondence to:

Thomas Kissel
Department of Pharmaceutics and Biopharmacy,
Philipps-University of Marburg
Ketzerbach 63
D-35032 Marburg, Germany
Telephone: (0049) 6421-2825880
Facsimile: (0049) 6421-2827016
E-mail: kissel@mail.uni-marburg.de

INTRODUCTION Restenosis is defined as a reobstruction of an artery following arterial interventions, such as balloon angioplasty, atherectomy, or stenting of an atherosclerotic plaque, and is characterized by intimal hyperplasia and vessel remodeling¹⁻⁴. Intimal growth results from vascular smooth muscle cell (VSMC) migration and proliferation into the media⁵ followed by the formation of extracellular matrix⁶. Currently, different routes of drug administration in restenosis therapy are under investigation. The proliferation of VSMC, the cause of restenosis development, could be inhibited by the application of radioactive⁷ and drug eluting stents^{8,9}. Another method to reduce VSMC growth and neointimal formation is the local administration of a drug solution using drug delivery catheters¹⁰; however, the delivery efficiency and intramural retention time of infused agents remains rather low^{11,12}. Therefore, researchers have developed colloidal drug carrier systems from biodegradable polymers that may provide a local release and sustained retention of the drug in the arterial wall¹³⁻¹⁷.

Recent studies have indicated that particle size plays an important role in the penetration and cellular uptake of particles into the vessel wall^{16,18}. Furthermore, particle size may even determine the biological response of the tissue to the foreign particle material^{19,20}. For instance, inflammatory reactions with subsequent fibrosis of vascular tissue have been found when applying particles with a size of 5 to 10 μm ^{13,21}.

Guzman et al²² observed that fluorescent-labeled particles of 165 nm that were first deposited in the luminal, medial, and adventitial layers of the artery were found afterward in only the adventitia. They concluded that the adventitial layer acts as a reservoir for particles and eluted drugs, which then subsequently diffuse in the direction of the media. A discontinuous particle distribution has been also found by Rome et al¹⁸. They showed that different types of particles, ranging between 90 to 500 nm in size, tended to accumulate in the outer wall layers but not in the media. These authors suggested that the particles reach the adventitia via the vasa vasorum, but not directly by penetrating the wall. Song et al^{23,24} explained the fluorescence activity of rhodamine B-loaded poly(D,L-lactide-co-glycolide (PLG) nanoparticles in the media of a dog carotid artery by the possibility of inward migration of nanoparticles from the

adsorption site at the intraluminal surface. On the one hand, these findings suggest that particles smaller than 300 nm are well suited as an effective intraluminal drug delivery system. On the other hand, the migration and deposition behavior of nanoparticles in the tissue is not yet clearly established.

The present study concentrates on the size-dependent penetration of nanoparticles into the aorta abdominalis of New Zealand white rabbits. In contrast to other authors, we used fluorescence-labeled instead of fluorescence-loaded particles to avoid any unintentional dye release that would prevent the exact determination of the particle distribution by confocal laser scanning microscopy (CLSM). Our investigations have shown that the particle migration through the wall tissue is in fact size dependent and, furthermore, is strongly influenced by the existence of atherosclerotic plaques at the luminal surface of the aorta, as well as by the formation of channel-like diffusion paths induced by pressurized infusion.

MATERIALS AND METHODS

Local nanoparticle delivery

All animal experiments followed the "Principles of Laboratory Animal Care" (National Institutes of Health publication #85-23, revised 1985) and were approved by an external review committee for laboratory animal care. Male New Zealand white rabbits (3.5-4 kg) were sacrificed using a mixture of embutramide, mebenzonium iodide, and tetracaine hydrochloride. The aorta abdominalis was exposed in situ (averaging 20-30 mm in length). For angioplasty and local delivery, a channeled balloon catheter (SCIMED REMEDY, Boston Scientific, Natick, MA) was placed in the aorta abdominalis. The delivery device has been described in detail by Hong et al.²⁵. Briefly, it is a 3-lumen over-the-wire catheter with separate ports for balloon dilation and local drug delivery. The balloon carries 18 channels, with 1 group of 30- μ m diameter pores per channel. The catheter shaft is 3.4 French. During this study, we used a balloon length of 20 mm and a diameter of 2.5 mm.

Yellow-green labeled polystyrene nanoparticles of 110, 217, and 514 nm in diameter (Fluoresbrite plain microspheres YG, Polyscience Inc, Warrington, PA) were suspended in water. Two milliliters of each suspension (0.025% (wt/vol) in water) were infused at a constant pressure of 2 atm and at a constant balloon pressure of 8 atm. A control experiment was performed by infusion of 0.9% sodium chloride solution. After the infusion experiments, arterial segments were removed and fixed in formalin solution (4% vol/vol) for CLSM or glutaraldehyde (2.7% vol/vol) in phosphate buffered saline (PBS) 0.1M, pH 7 for transmission electron microscopy.

Confocal laser scanning microscopy

Arterial segments were frozen in isopentane on ice and embedded in Tissue Freezing Medium (Jung, Germany). Cross-sections of 20- μ m thickness were cut using a Frigocut 2700 cryo microtome (Reichert-Jung, Germany) and mounted on SuperFrost plus (Menzel-Gläser, Germany) glass slides. They were counterstained with 4'6-diamidino-2-phenylindole DAPI (1 μ g/mL) (Molecular Probes, Leiden, The Netherlands) solution in PBS for 30 minutes under light exclusion and embedded in PBS/glycerol (2:1 vol/vol). Localization of fluorescence-labeled nanoparticles was performed using a CLSM (Axiovert, Zeiss CLSM 501, Jena, Germany) equipped with a Zeiss Neofluor 40*/1.3 objective. Excitation wavelengths were 364 nm (long-pass filter [LP] 385 nm) for DAPI and 488 nm (LP 505 nm) for the yellow-green labeled nanoparticles. All confocal images were acquired with the same settings with respect to laser intensity, filter block, and detector gain.

Transmission electron microscopy

The fixed blood vessels were rinsed 3 times with 0.15M PBS, pH 7 for 1 hour each and postfixed for 75 minutes in 2% osmium tetroxide. Dehydration was accomplished in a series of mixtures of water with component A (a water-soluble aliphatic polyepoxide) of the Durcupan ACM water-soluble embedding medium (Fluka, Germany) as follows:

- 50% component A with 50% water for 30 minutes
- 70% component A with 30% water for 45 minutes
- 90% component A with 10% water for 45 minutes
- 2 x 100% component A for 90 minutes

The dehydrated tissue was then placed in a polymerization mixture of the components A through D (components B and C are hardeners; component D is the plasticizer) according to the manufacturer's protocol and left overnight in a refrigerator for final mixing and embedding. Polymerization was performed in a freshly prepared mixture of the above composition for 4 days at 42°C.

Ultrathin sections, about 50-nm thick, were obtained using a Leica Ultracut ultramicrotome (UCT) and a diamond knife (Leica Microsystems, Bensheim, Germany). The sections were collected on copper grids covered by a thin layer of colodium and carbon. Final staining of the sections included treatment with uranylacetate for 15 minutes and with lead citrate for 9 minutes. Microscopic examinations were carried out with a Joel Electron Microscope (JEM) 3010 transmission electron microscope (Jeol Electron Microscope, Japan)

Figure 1. CLSM analysis of the nanoparticle distribution in the vessel wall of the aorta abdominalis of New Zealand white rabbits: nuclei (blue) stained with DAPI, catheter-delivered yellow-green labeled polystyrene nanoparticles (green). P, L, M, and A indicate the atherosclerotic plaque, lumen, media, and adventitia. (A) Balloon-dilated segment of aorta abdominalis: control experiment without nanoparticle infusion. (B) Confocal image: accumulation of 514-nm particles on the luminal surface of the aorta. (C and D) Confocal images: distribution of 217-nm particles in the aorta abdominalis; optical sections were taken from the inner medial (C) and the adventitial part (D) of the vessel wall layers (2.87 μm each layer).

operated at 300 kV, equipped with MegaScan charge-coupled device (CCD) camera (2048 x 2048 pixel).

Results Cross-sections from the infusion experiments without fluorescent probes exhibited no or negligible green autofluorescence at standard confocal settings (**Figure 1A**). Green autofluorescent emissions of arterial elastin and collagen structures can be well distinguished from the blue stained nuclei of smooth muscle, endothelial, and adventitial cells, as well as from the nanoparticles. The observed cross-sections of the arterial segments subjected to intramural nanoparticle infusion using a perforated balloon catheter did not show any severe disruptions of the vessel wall.

Confocal laser scanning analyses revealed a size-dependent penetration depth of the nanoparticles in the nonatherosclerotic arterial wall at constant infusion pressure. As displayed in **Figure 1B**, the 514-nm nanoparticles were observed to accumulate preferentially at the luminal surface of the aorta. Only a negligible number of particles was detected in the inner parts of the vessel wall. With decreasing diameter, the penetration capability of the particles increased, as shown in the case of 217-nm particles in Figures 1C and 1D, and 110-nm particles in **Figure 2A**. High fluorescent activity in deeper cell layers of the vessel wall, especially

Figure 2. Confocal images: distribution of 110-nm particles in a nonatherosclerotic vessel segment (A) in comparison to an atherosclerotic segment (B).

in the latter case, indicates that nanoparticles of 110 nm easily penetrate through the arterial wall. Sometimes a tendency toward cluster formations was observed on the luminal surface. Furthermore, it seems quite obvious that the particle distribution between the luminal site and the adventitial layer is not continuous. This is documented by systematic observations and is not based on sectioning artefacts. On the other hand, discontinuous particle concentrations crossing the artery wall may be caused by differences in the accessibility, the transmission properties, and the holding behavior of the different vessel wall layers.

While the CLSM images provide an overview of the delivery characteristics of the nanoparticles across the blood vessel, the precise localization of the particles within a particular tissue layer needs a higher resolution achieved by using transmission electron microscopy. Figures 3A to 3F are transmission electron micrographs of stained ultra-thin sections from the endothelial region (Figures 3A-3C) and the media (Figures 3D-3F) for the 3 particle sizes used: 514, 217, and 110 nm. In the inner region the endothelial cells show mechanical distortion due to the catheter intervention. In the medial layer typical views of the glycogen strands are revealed. The size dependency of particle deposition is proved by Figures 3A to 3F, with only the small-sized particles reaching the inner wall regions in considerable amounts.

Observations of atherosclerotic arteries showed that plaques on the endothelial cell layer are able to inhibit even the penetration of the 110-nm nanoparticles (**Figure 2B**). The plaque consists of collagen, fibrin, smooth muscle cells, foam cells, and macrophages, which form a rather solid layer on the inner vessel surface. Hence, they may act as a strong barrier to particle penetration. Preliminary experiments using nanoparticles as small as 57 nm in diameter have led to the same result (data not shown): hindrance of transportation into the vessel wall by a barrier of extended plaque formation.

The elastic connective tissue can also function as a strong transport barrier for the nanoparticles, as **Figure**

Figure 3. Transmission electron micrographs of the accumulation of 110-, 217-, and 514-nm particles in the endothelial layer (A, C, E) and in the media (B, D, F) of the aorta abdominalis of New Zealand white rabbits (lumen left). Segment D provides clear proof of how the elastica tissue acts as a diffusion barrier.

Figure 4. Scanning electron micrographs of the infusion holes in a REMEDY balloon catheter showing one group comprising 18 channels (A) and 2 holes at higher magnification (B). Some holes remained filled with organic material after use.

3D illustrates. The 217-nm particles are strung like pearls alongside the interface to the elastica externa; the particles were neither able to penetrate nor to permeate the elastic tissue.

The CLSM image in **Figure 1C** is part of a gallery of 7 optical sections (2.87 μm each section). In one of the upper sections of this sequence, particle deposition in the endothelium and the adventitia was observed, as was particle deposition along a radial channel-like trace connecting the inner and outer regions of the vessel, as **Figure 1D** shows. It is suggested that such traces originate from the directed stream of nanoparticles ejected under pressure through the holes of the perforated balloon catheter. The size and the configuration of the infusion holes in the catheter wall

may, thus, influence the distribution characteristics of the nanoparticles in the blood vessel strongly. **Figure 4** shows the typical arrangement and shape of the holes in the REMEDY catheter used in this study. The 25- μm holes are arranged in groups of 18, of which 6 such groups are distributed over the circumference in a repetitive manner at a distance along the catheter axis of approximately 5 mm. The hole diameter and the width of a diffusion channel in **Figure 1C** are in the same order of magnification.

DISCUSSION The feasibility of local drug delivery with a perforated balloon catheter for the prevention of restenosis was first demonstrated by Wolinski and Thung¹⁰. However, the intramural delivery efficiency and

the retention time of liquid agents was low¹⁰⁻¹³. To overcome this drawback, microparticles have been evaluated as potential drug delivery vehicles¹³⁻¹⁷. The predominant sites of microparticle deposition were found to be the adventitial vasa vasorum, the periadventitial microvasculature, and along intimal disruptions^{16, 17} but usually not the target tissue, the media. One important aspect of these carrier systems is the effect of particle size on penetration behavior and cellular uptake by the vessel wall¹⁸. In addition to that, particle size is associated with biological response of the tissue to foreign biomaterials^{19, 20}. While inflammatory reactions with subsequent fibrosis is a typical response of vascular tissue to particulate delivery systems ranging in size between 5 and 10 μm ^{13, 21}, nanoparticles usually cause little or no focal inflammation.

Guzman et al²² found fluorescence-loaded PLGA particles of 90 to 250 nm predominantly at the lumen surface of the vessel wall and in the adventitial layer. Only a few particles were observed in the media²². Rome et al¹⁸ suggested that particles smaller than 500 nm, which had mainly accumulated within the intimal and adventitial layers, reach the media via vasa vasora of the artery. Song et al²³ reported fluorescence activity of 100 to 200 nm rhodamin B-loaded PLGA nanoparticles in the media of a dog carotid artery, which was explained by the possible migration of particles from adsorption sites at the luminal surface.

Our study adds an additional aspect to the understanding of particle migration. The channel-like deposition patterns observed after application of 217-nm particles seem to be caused by the pressure-induced particle stream from the catheter pores through the intima and media into the adventitial layer. To achieve a similar effect using larger particles, the infusion pressure needs to be increased considerably. This could have detrimental consequences to the wound healing process of the injured vessel wall. When smaller nanoparticles (=200 nm) are applied, a lower infusion pressure is necessary; thus, the formation of extended vessel lesions is prevented. The most favorable pressure level should provide sufficient nanoparticle deposition in the vessel wall and, at the same time, avoid unrecoverable vessel irritations. According to our experience, pressure levels of about 2 to 4 atm are efficient and atraumatic in this model.

Possible anatomic barriers to any particle transportation through the wall tissue are the intact internal and external elastic laminae. Moreover, the presence of atherosclerotic plaques on the inner luminal surface strongly influences the particle penetration into the arterial wall, as our results have shown. Independent of their size, nanoparticles are deterred at the surface of the dense plaque layer and are unable to penetrate the vessel wall. This observation points to additional complications of drug delivery strategies in restenosis.

Because of their size, nanoparticles are suitable as drug carrier systems for localized application of therapeutic agents in restenosis therapy. In the present study, the local administration of polystyrene nanoparticles using a perforated balloon catheter could be successfully visualized by CLSM and transmission electron microscopy. It was demonstrated that nanoparticles can penetrate the nonatherosclerotic arterial vessel wall and that the penetration depth can be adjusted by varying the particle size. Direct evidence is provided for the existence of very local penetration paths of the nanoparticles as result of the pressure-directed particle stream during balloon infusion.

Apart from anatomic barriers—including the intact endothelial layer, as well as intact internal and external elastic laminae—atherosclerotic plaques seemed to prevent nanoparticulate drug carriers from penetrating the vessel wall in the absence of dissections. Further experiments on the effect of infusion pressure, suspension volume, and nanoparticle concentration are under way to clarify in detail the suitability of nanoparticles as effective intraluminal drug delivery systems for the prevention of restenosis after balloon angioplasty.

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