Quantum dots exhibit unique optical properties for bioimaging purposes. We have previously developed quantum dots with a paramagnetic and functionalized coating and have shown their potential for molecular imaging purposes. In the current mini-review we summarize the synthesis procedure, the in vitro testing and, importantly, the in vivo application for multimodal molecular imaging of tumor angiogenesis.

**Keywords** Quantum dots · Molecular imaging · Angiogenesis · Multimodal imaging · Magnetic resonance imaging · Fluorescence imaging · Intravital microscopy

**Introduction**

Quantum dots (QDs), semiconductor nanocrystals, have exceptional optical properties, which make them ideally suited for a number of applications in biomedical imaging, with several important advantages over fluorescent dye-molecules [1, 2]. The absorption spectrum of a QD is very broad, while the emission spectrum is narrow. The exact optical properties of QDs can be tuned by a precise control of composition and size, allowing the synthesis of QDs with emission wavelengths spanning from the near UV to near infrared (NIR), making QDs particularly suitable for multiplexed imaging [3]. Importantly for biological applications, the surface of QDs can be easily modified to render them water-soluble and biocompatible as well as to allow the introduction of additional functionalities and to make them target-specific. Dubertret and colleagues were the first to apply quantum dots to a living organism (Xenopus embryos) for in vivo imaging purposes [4]. To that end they encapsulated the QD nanocrystals in phospholipid micelles. Previously, we modified their procedure to create QD-based nanoparticles that can be detected by optical techniques as well as magnetic resonance imaging (MRI). Using this approach we have created QD based nanoparticles that specifically bind activated endothelial cells to image ongoing angiogenesis [5, 6].

**Synthesis of paramagnetic QD-micelles**

The original procedure involves mixing of QDs that are coated with hydrophobic capping molecules with an excess of PEGylated phospholipids (PEG-lipids) and Gd-DTPA labeled lipids (Gd-DTPA-DSA) in chloroform. Upon evaporation of the solvent, a mixed film containing the lipids and QDs is formed. Hydrating and heating this film results in the formation of paramagnetic micelles that contain a QD in their core. This procedure results in the formation of a relatively large fraction of aggregated QDs coated by the PEG-lipids. To increase the yield of paramagnetic micelles with a single QD core we modified the procedure as illustrated in Fig. 1. The new method involves...
slow infusion of the chloroform QD/lipid mixture into hot water, which results in the formation of chloroform-in-water emulsions that swiftly form micelles as chloroform evaporates. The resulting mix of QD core micelles and empty micelles is separated by centrifugation methods. The relaxivity of purified paramagnetic QD-micelles is approximately 2,000 mM$^{-1}$s$^{-1}$ (per mM nanoparticles) at a clinically relevant field strength of 1.41 T, which makes them attractive for molecular MRI purposes [7, 8].

By the inclusion of maleimide functionalized PEG-lipids (Mal-PEG-lipid), the paramagnetic QD-micelles can be functionalized via a sulfhydryl-maleimide coupling method. Using this strategy, we have shown the conjugation of $\alpha_v\beta_3$-integrin specific cyclic RGD peptides [5, 9] and phosphatidylserine specific Annexin A5 proteins [6] to the paramagnetic QD-micelles, but also the conjugation of E-selectin specific antibodies to paramagnetic liposomes [10].

**In vitro targeting and imaging**

To assess the biological specificity of the RGD and Annexin A5 conjugated paramagnetic QD-micelles (abbreviated as RGD-pQD and A5-pQD, respectively) human umbilical vein endothelial cells (HUVEC) and Jurkat cells were used for in vitro testing, respectively. Growth factor activated and proliferating HUVEC overexpress the $\alpha_v\beta_3$-integrin, while Jurkat cells exposed to anti-Fas become apoptotic and expose the negatively charged phospholipid phosphatidylserine (PS) at the outer layer of the cell membrane. Both markers are known to be expressed in angiogenic blood vessels. In Fig. 2a a T$_1$-weighted MR image of loosely packed cells, 1.5 million per pellet, is shown. The pellet of cells incubated with the targeted RGD-pQDs appeared much brighter than those that were incubated with non-targeted pQDs or that were not incubated with contrast agent. A fluorescence microscopy image of HUVEC that had been incubated with green emitting RGD-pQDs is depicted in Fig. 2a (right) and showed the nanoparticles to be internalized, at a perinuclear location (inset). In Fig. 2b Jurkat cell pellets are shown that were illuminated with 254 nm UV light. The green emitting cell pellet originating from the A5-pQDs incubated PS expressing cells can be clearly distinguished from the control cell pellets.

**Multimodality molecular imaging of tumor angiogenesis**

To evaluate the in vivo potential of RGD-pQDs we designed a study where different groups of tumor bearing mice were imaged in vivo with fluorescence intravital microscopy (IVM), MRI, or whole body fluorescence imaging (Fig. 3, left) [9]. IVM was used to monitor nanoparticle binding to the tumor vasculature in real-time. We observed labeling of tumor blood vessels within 5–10 min after the administration of RGD-pQDS. A typical fluorescence image is depicted in Fig. 3. Labeling of endothelial cells in the tumor vasculature by RGD-pQDs was found as far as 1 cm from the tumor periphery, indicative for a widespread activation of the blood vessels in close proximity of cancerous tissue. No endothelial cell labeling of blood vessels in the hind limb of the animals and of blood vessels in the ears of the animals was observed.

MRI was performed on a different group of animals that were intravenously injected with RGD-pQD. A high resolution T$_1$-weighted image, acquired 45 min after contrast agent injection, revealed angiogenic activity to be mainly found at the rim of the tumor, which corresponds with the regions of the tumor with highest angiogenic activity.

Lastly, whole body fluorescence imaging was employed to visualize angiogenesis in nude mice that grew a tumor in their kidney (Fig. 3, bottom). This technique has a higher sensitivity and temporal resolution than MRI and thus allows faster screening of nanoparticle targeting, albeit
Altogether, IVM, MRI and fluorescence imaging are complementary imaging modalities that, when combined with our nanoparticle, can provide information about angiogenesis at the microscopic level of superficial blood vessels (IVM), at the whole mouse level with high sensitivity and temporal resolution (fluorescence imaging), as well as three dimensional information of the inside of the mouse and tumor (MRI).

Discussion and conclusion

QDs represent an excellent tool for multimodal molecular imaging of angiogenesis. It was recently demonstrated that these nanocrystals can be used to image the tumor micro-environment and that differently sized quantum dots can be used to assess the permeability of the tumor microvasculature [11]. Subsequent to the lipid-based QD platform we introduced, Oostendorp and colleagues demonstrated the functionalization of QDs with paramagnetic dendritic wedges and CD13 specific cNGR peptides to allow MR molecular imaging of tumor angiogenesis [12] as well as angiogenesis after myocardial infarction [13]. The fluorescence properties of the QDs were exploited to validate their findings with ex vivo two-photon laser scanning microscopy, which showed the QDs to be co-localized with endothelial cells. In addition to labeling QDs for MRI, studies have shown the value of the combination with positron emission tomography (PET) imaging. Cai and colleagues used radiolabeled and RGD peptide function-alized QDs to accomplish molecular imaging of tumor angiogenesis [14], while Chen et al. demonstrated the same using radiolabeled QDs that were functionalized with vascular endothelial growth factor [15]. Since our nanoparticle platform contains DTPA functionalized lipids the inclusion of a radiolabel should be relatively straightforward and may be introduced as a third label to additionally allow PET imaging. Interestingly, the flexibility of the QD-micelle platform also allows the possibility to include therapeutics [16] or additional targeting moieties to enhance the specificity of targeting, as was recently demonstrated by Kluza et al. for a liposomal nanoparticle [17].

In conclusion this mini-review presented the development of paramagnetic QD-micelles for MR and optical based molecular imaging. The ease of preparation and flexibility, as well as their use as a scaffold that is representative of other nanocrystals make them fruitful tools for biomedical imaging purposes. Their application may be
extended to other imaging modalities, to other disease processes than angiogenesis, as well as for drug targeting purposes.

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