COBALT COMPLEXING ABILITY OF HYDROSOLUBLE POLYAMIDOAMINES USED AS NON-VIRAL VECTORS FOR INTRACYTOPLASMIC DELIVERY

Luigi Falciola, Paolo Ferruti, Beatrice Malgesini, Nadia Missaglia, Patrizia Mussini, Elisabetta Ranucci, Manuela Rossi

Introduction

Amphoteric polyamidoamines, an important class of hydrosoluble polymers for biomedical applications[1-4]

One of the most exciting frontiers of macromolecular science in the new millennium lies in widening the use of biomedical polymers and polymer therapeutics. Functional polymers have found many applications, for instance as ion-exchange resins, as well as agents for the surface modification of heparin filters and non-thrombogenic surfaces [1-4]

Polymer therapeutics include polymeric drugs, polymer drug conjugates, polymeric micelles entrapping drugs by covalent linkage, as well as bioresponsive polymers used as components of non-viral vectors for intracellular delivery. These systems are just entering early clinical evaluation.

Polyamidoamines (PAAs) are a unique family of synthetic functional polymers that have been widely developed for use both as biomedical materials and polymer therapeutics. PAAs are water-soluble and designed to be biodegradable and biocompatible [3,5]. They are easily functionalised to provide sites for the attachment of drug residues and targeting moieties[6]. Since they display intelligent "stealth" properties they are currently under development as PAA/anticancer conjugates and non-viral vectors for intracytoplasmic delivery.

The latest developments in PAAs science

It is possible to obtain amphoteric PAAs whose acid-base properties are tailored to meet specific requirements. For instance, PAAs containing two weak ter-amino groups and a single strong carboxyl group per repeating unit are prevailingly anionic in extracellular fluids, where the pH is 7.4. When injected in animals, these PAAs exhibit remarkable "stealth" properties, and concentrate specifically in tumour tissues by the "enhanced permeation and retention" (EPR) effect [7].

The same PAAs, however, increase their polycationic character and display pH-dependent membrane-disruptive properties by lowering the pH, being most lytic at pH 5.5 [8].

After internalisation in cells by pinocytosis, amphoteric PAAs, like many other polymers entering cells from extracellular fluids, are localised in lysosomes. As inside lysosomes the pH is 5.5 or whereabouts, these PAAs after internalisation fully exhibit their membrane-disruptive properties that were latent while circulating in extracellular fluids. Therefore, PAAs have the potential to act as a synthetic alternative to fusagenic peptides, thus promoting endosomal escape.

We have found that PAAs have the ability to complex DNA, to protect it from nuclease degradation, and to promote transfection *in vitro*. PAAs demonstrated the ability to mediate pSV β–galactosidase transfection of HepG2 cells. At a vector/DNA mass ratio of 5 : 1, an amphoteric PAA dubbed ISA 23 showed equivalent transfection ability compared with polyethyleneimine and "LypofectIN" proving more effective than "LypofectACE". We may reasonably conclude that the unique combination of properties exhibited by amphoteric pAAs foster their development both as polymeric drug carriers and endosomolytic vectors for intracytoplasmic delivery of genes and toxines [9,10].

Recently, the influence of counterions on endosomolytic properties as a function of pH of several PAA salts has been investigated, choosing as a model their ability of inducing red blood cell lysis at different pH values. The hydrochlorides, sulphates, phosphates, lactates, citrates, and acetates of typical PAAs named ISA 1, ISA 4, ISA 22, and ISA 23, were selected for this study. None of the PAA salts were haemolytic at pH 7.4 or pH 7.1 (at 1 h), and the acetate, citrate, lactate, and phosphate forms of ISA 1 and 4 were also non-haemolytic at pH 5.5 (1 h). In contrast, the ISA 1

sulphate, the ISA 4 chloride and sulphate, and the ISA 22/23 chloride, sulphate and phosphate forms showed significant haemolysis at pH 5.5 (>50% haemolysis at 1 h). Haemolysis was time-dependent, and indeed the PAAs showed considerably more haemolysis than the polyethyleneimine (PEI) reference control (50-90% haemolysis for PAAs when compared with <20% for PEI and dextran). All the PAAs were relatively not toxic (IC_{50} > 1 mg cm^{-3}). Although ISA 4 was cytotoxic for B16F10 and ECV-304 cells at concentrations > 1 mg cm^{-3}, the different PAA salt forms where equally toxic.

Ion-complexing properties of PAAs

Bearing amino and carboxylic functional groups such as the well-known metal ion complexing agent EDTA, the PAAs are able to form coordination complexes with heavy metal ions such as Cu^{2+}, Ni^{2+}, Co^{2+}, and several complexation constants are reported e.g. for copper complexes of several PAAs in Ref. [3]. Studying cobalt complexation is particularly attractive from the biomedical point of view, because it is related to the possibility of introducing radioactive isotopes inside tumoral cells for diagnostic and curative purposes.

Experiment

We performed EXAFS measurements on **Co**$^{2+}$ in the following samples:

1. **Co**Cl_2 0.05 M in water (as a standard)
2. EDTA + **Co**Cl_2 1: 1 (0.05 M) in water, as such, and at controlled pHs = 3,5,7 and 9
3. PAA1 + **Co**Cl_2 1: 1 (0.05 M) in water, as such, and at controlled pHs = 3,5,7 and 9
4. PAA2 + **Co**Cl_2 1: 1 (0.05 M) in water, as such, and at controlled pHs = 3,5,7 and 9
5. solid PAA1 membrane ("hydrogel" phase, conditioned in **Co**$^{2+}$)
6. solid PAA2 membrane ("hydrogel" phase, conditioned in **Co**$^{2+}$)

Cobalt K-edge (7709 eV) EXAFS sorption spectra were recorded in the fluorescence mode at BM8-GILDA beamline, European Synchrotron Radiation Facility (ESRF), Grenoble, France.
Fluorescence measurements were performed at room temperature and pressure with a Si (311) double-crystal monochromator and a 15-element solid-state detector.
Samples 1 to 4, being solutions, required the special Teflon cell with Kapton windows available in GILDA's laboratories, while samples 5 and 6 were two square hydrogels, 2 x 2 cm in dimension, which were previously conditioned for 12 hours in a 0.05 M and 0.1 M Co^{2+} solution, in order to reach sorption equilibrium. Hydrogel surfaces were dried with paper, then inserted between two Kapton-tape foils and then placed in the experimental hutch. Kapton-tape was previously tested for giving no absorption in the experimental energy range investigated.

Qualitative and quantitative structural information for the Co^{2+} environment were obtained using the GnXAS Analysis Program which applies a fitting procedure in order to obtain the best fit between the theoretical and experimental spectrum. The X-ray absorption spectra were background-subtracted in the EXAFS region by fitting a straight line through the pre-edge region and a set of two splines beyond the adsorption edge, and thus normalized using the height of the edge-step near the maximum sorption edge.

Results

The structure of the coordination sites was defined by reference to a literature case, notably the authoritative work by D'Angelo et al. [11], which deals with the analysis of Co^{2+} aqueous solutions. In that work, the best fit was obtained under the assumption of a regular octahedral Co^{2+} aquocation structure featuring six O atoms at 2.06±0.03 Å, notwithstanding many alternative attempts with assumptions of different coordination distances *i.e.* distorted octahedral geometries. In our case, a comprehensive analysis was complicated by the fact that it is almost impossible, in

the EXAFS spectra, to discriminate between O and N atoms surrounding the central cobalt atom, due to their proximity in the periodic table. Nevertheless, our results indicated an asymmetric octahedral cobalt coordination structure. The asymmetric structure resulting from data analysis is consistent with the involvement in cobalt coordination of two different functional groups, and, in fact, the ISA23-NH2-BAC repeating unit (Figure 1 (b)) includes a piperazine ring with two aminic nitrogen atoms in the 1,4 positions, which are known to give simultaneous coordination with the ring in the boat conformation[12], plus a carboxylic group providing a further coordinating oxygen atom at a suitable distance. Therefore, cobalt coordination could be attained involving two ISA23-NH2-BAC repeating units, probably not closely adjacent in the hydrogel network, each one contributing to the complex with two nitrogen atoms from the boat-conformed piperazine ring plus one oxygen from the carboxylate group. Incidentally, the above optimized Co/heteroatom distances are consistent with similar cases of Co(II) complexation, such as the 2.13 Å value found in cited work [13] for most of the coordinating O and N atoms.
It is also worth mentioning that our work is, to our knowledge, the first application of EXAFS spectroscopy to the study of metal ion coordination in hydrogel phase.

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