

SHORT REPORT

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Extended blood circulation and joint accumulation of a p(HPMA-co-AzMA)-based nanoconjugate in a murine model of rheumatoid arthritis

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Abstract

Background: We recently synthesized a hydrophilic polymer, poly(*N*-(2-hydroxypropyl)methacrylamide-co-*N*-(3-azidopropyl)methacrylamide), p(HPMA-co-AzMA), by RAFT polymerization using a novel azide-containing methacrylamide monomer that through a post modification strategy using click chemistry enabled facile preparation of a panel of versatile and well-defined bioconjugates. In this work we screen a panel of different molecular weight (Mw) fluorescently tagged p(HPMA-co-AzMA) in healthy mice, by live bioimaging, to select an extended circulatory half-life material for investigating joint accumulation in a murine collagen antibody-induced arthritis model.

Findings: Fluorescence image analysis revealed half-lives of <20 min, 2.8 h and 6.4 h for p(HPMA-co-AzMA) of 15, 36 and 54 kDa, respectively, with ~10% polymer retained in the blood after 24 h for the highest Mw. p(HPMA-co-AzMA) of 54 kDa showed enhanced accumulation in the joints of the arthritic mouse model with a bioavailability (AUC = 1783% · h) ~12 times higher ($P = 0.01$) than healthy control (AUC = 148% · h).

Conclusions: p(HPMA-co-AzMA) of 54 kDa exhibited extended circulatory half-life and preferential accumulation in inflamed joints of a murine model of rheumatoid arthritis (RA). This combined with well-defined polymer size and versatility for conjugation of a range of biomolecules promotes p(HPMA-co-AzMA) for potential applications in the delivery of drugs for treatment of RA.

Keywords: Arthritis, Collagen Antibody-Induced Arthritis, Extended circulation, HPMA, *in vivo*, Image analysis, Joint accumulation, *N*-(3-azidopropyl)methacrylamide, Poly(*N*-(2-hydroxypropyl)methacrylamide)

Findings

Introduction

Rheumatoid arthritis (RA) is a chronic progressive autoimmune disease affecting ~ 1% of the population causing cartilage and bone destruction in synovial joints [1]. The pathogenesis involves synovial infiltration by circulatory immune cells that induce inflammation, modulated predominantly by proinflammatory cytokines such as tumor necrosis factor alpha (TNF α) [2,3]. Common clinical treatments include nonsteroidal anti-inflammatory drugs (NSAIDs), glucocorticoids, and disease-modifying anti-rheumatic drugs (DMARDs) such as anti-TNF α antibodies.

Although effective in many patients, these therapeutics have associated side-effects such as myelosuppression and increased infection risk, primarily due to a general systemic immune suppression that necessitates alternative approaches [1,4]. RA associated angiogenesis, necessary for formation of pannus and invasion of inflammatory cells into the synovial tissue, has been identified to establish a macromolecular retention effect [5-7], conceptually similar to the EPR effect for tumors [8]. Hydrophilic polymers synthesized with high molecular weights (Mw) enable the construction of polymer macromolecular drugs [9] with extended blood circulation and specific targeting to arthritic joints. This has been used for local suppression of proinflammatory cytokines such as TNF α [10] that may increase the clinical efficacy and reduce generalized side effects.

The field of polymer therapeutics requires increased attention to defining and minimizing macromolecule

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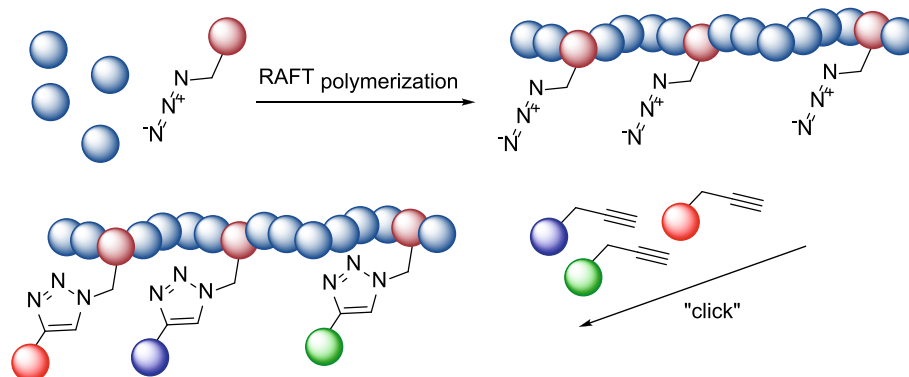


Figure 1 Synthesis and functionalization of the p(HPMA-co-AzMA) copolymer as a versatile method for production of polymer bioconjugates (Reproduced from ref. 13 with permission from John Wiley and Sons).

heterogeneity and polydispersity due to its great influence on pharmacokinetics, safety and efficacy of polymer therapeutics and to identify precise polymer characteristics required for delivery e.g. to RA affected tissue [11,12]. We have recently synthesized a novel azide containing copolymer, poly(*N*-(2-hydroxypropyl)methacrylamide-co-*N*-(3-azidopropyl)methacrylamide), (p(HPMA-co-AzMA)), through a versatile post-modification procedure to produce a panel of well-defined polymer bioconjugates of narrow PDI (Figure 1) [13]. Compared to existing bioconjugation strategies for RA [14,15], this may provide superior polymer characteristics in terms of flexibility for modification and low polydispersity.

Research hypothesis

We hypothesize that the p(HPMA-co-AzMA) constructs will exhibit prolonged blood circulation and specific accumulation in inflamed joints in a murine model of rheumatoid arthritis supporting its potential therapeutic application for RA.

Methods

For blood circulation studies, a PBS solution (100 μ L) of p(HPMA-co-AzMA) polymers (4 mg/kg) of different Mw (15, 36 and 54 kDa) labeled with an Atto680 dye (ATTO-TEC GmbH, Siegen, Germany), or PBS only control was administered intravenously (i.v.) in healthy BALB/c male mice (8 week-old, Taconic Europe, Ry, Denmark). Blood volumes (\sim 80 μ L, max 2 pr. animal) were sampled from 5 min up to 24 h post-injection and the blood plasma was subsequently transferred to narrow 20 μ L glass capillary tubes (minicaps, Hirschmann) and imaged with the IVIS[®] Spectrum using a 675/720 nm filterset and analyzed using the Living Image software version 4.3 (PerkinElmer).

Nine week-old, male, DBA/1 (Taconic Europe, Ry, Denmark) were used for the collagen antibody-induced arthritis (CAIA) model [16] with intraperitoneal (i.p.) injection of the monoclonal antibody mix (Arthrogen-CIA[®]

arthritogenic monoclonal antibody 5 clones cocktail kit, Chondrex, Inc. Redmond, USA) and a subsequent LPS injection 3 days later. The severity of the arthritic condition was monitored by daily clinical scoring of joints (1–4) [16] with symptoms developing \sim day 3 and peaking around day 8. Healthy control mice (DBA/1) were injected i.p. with PBS.

For the joint accumulation studies, a PBS solution of the 54 kDa Atto680-labeled (0.57 w/w%) p(HPMA-co-AzMA), or the free dye, was administered by intravenous (i.v.) injection to arthritic and control animals at a polymer dose of 4 mg/kg or the dye-equivalent amount (22.6 μ g/kg) of free dye. This dose was comparable to existing studies [17,18] and at a concentration by which the polymer conjugated dye could be easily visualized using *in vivo* fluorescence imaging. Joint fluorescence was monitored before, and up to 24 h post-injection by IVIS Spectrum

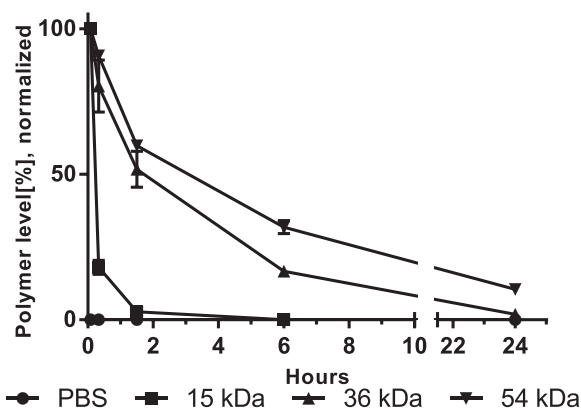
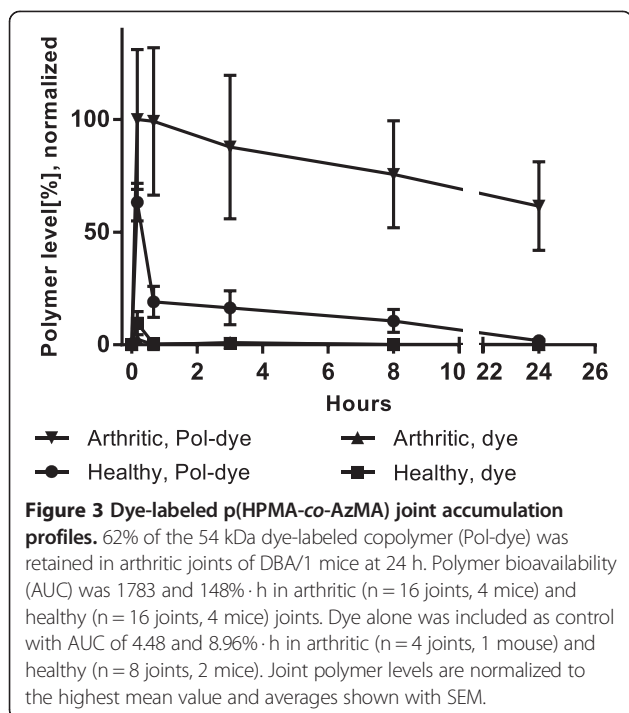


Figure 2 Blood circulation profiles. Blood circulation profiles of dye-labeled p(HPMA-co-AzMA) of 15, 36 and 54 kDa (PBS as control) in BALB/c mice. Polymer levels were normalized to the value measured at 5 min. 10% of the 54 kDa polymer was retained in the blood at 24 h. Polymer bioavailability (AUC) are 698, 313 and 45% \cdot h for polymers of 54, 36 and 15 kDa, respectively. Polymer circulation half-life was calculated to 6.4, 2.8 and $<$ 0.33 h for polymers of 54, 36 and 15 kDa, respectively. Averages of n = 3 mice are shown with SEM.



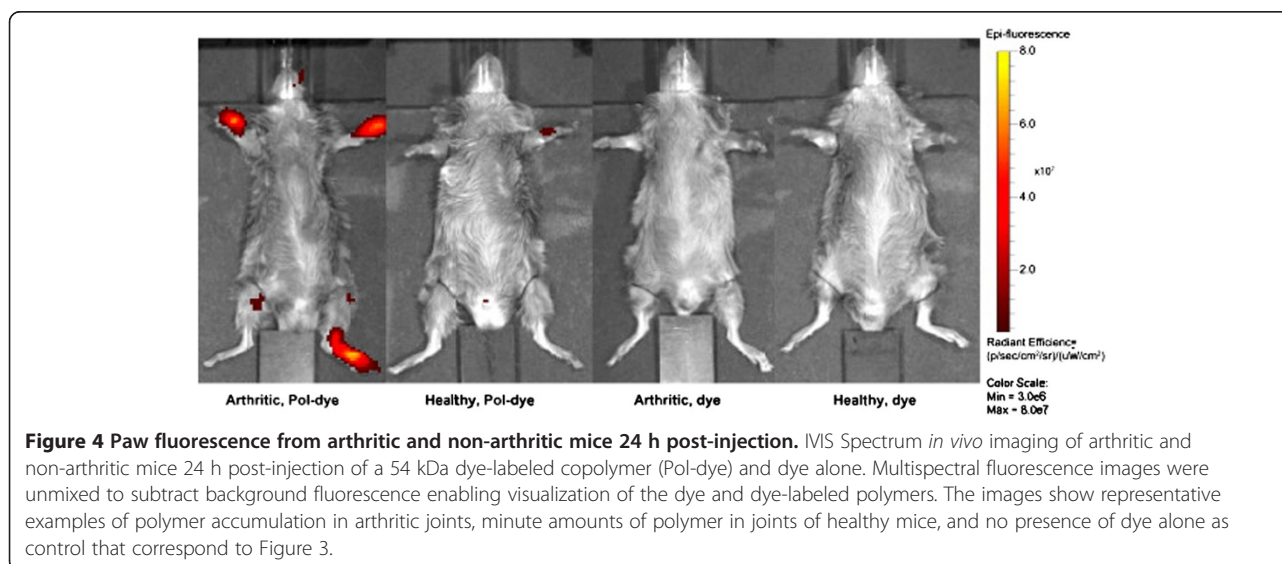
in vivo imaging of isoflurane (2.5%) anesthetized mice. Multispectral fluorescence images covering the absorption-emission profile of the Atto680 dye were acquired using the Living Image software (PerkinElmer). Images were spectrally unmixed to subtract background fluorescence, which enabled the total fluorescence emission from joint areas from each mouse to be quantified. Measured intensities of the fluorescence emission are generally linear dependent on fluorophore concentration, extinction coefficient and quantum yield, which enables a direct correlation to polymer levels in the blood or tissue [19].

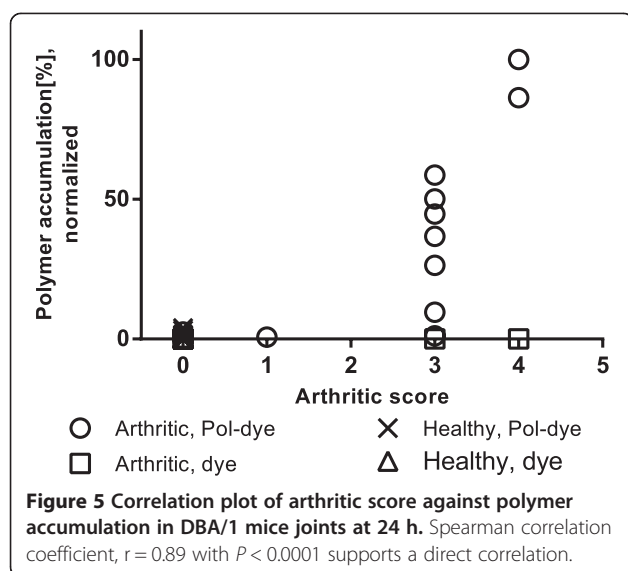
All image data was analyzed using Prism (GraphPad Software Inc.). Blood polymer levels were normalized to the value measured at 5 min and joint polymer levels to the highest mean value and presented as % with SEM. Students t-test was performed to determine data significance.

All procedures of animal work were performed according to international recognized guidelines and the animal experimental protocols approved by ‘The Experimental Animal Inspectorate in Denmark’ under The Danish Veterinary and Food Administration, Ministry of Food, Agriculture and Fisheries (Registration number: 2013 – 15 – 2934 – 00789-C2, issue date: March 5, 2013).

Results and discussion

Blood circulation profiles (Figure 2) constructed from blood plasma fluorescent polymer levels showed a Mw-dependent prolonged blood retention of the copolymers. At 24 h, the 54 kDa copolymer was still retained in the blood at ~10% and accordingly displayed a significant ($P < 0.03$) higher bioavailability (area under the curve, AUC = 698% · h) compared to 36 kDa (AUC = 313% · h) and 15 kDa (AUC = 45% · h) polymers [see Additional file 1: Figure S1]. First order clearance kinetics were modeled from 1 h post i.v. injection of dye-labeled copolymers of different Mw and exhibited a half-life of < 20 min for polymers of 15 kDa that increased up to 2.8 h for 36 kDa and 6.4 h for 54 kDa polymers, in general accordance with previous studies on HPMA copolymers [18]. Renal filtration commonly occurs for polymers with a hydrodynamic radius below 5 nm that corresponds to ~30 to 50 kDa Mw, however, with renal clearance of linear polymers, such as in this work, being up to 10 times higher than those of more globular-shape [20]. In this work, the highest Mw p(HPMA-co-AzMA)





did not seem to be excessively larger than the renal clearance limit, but still exhibiting prolonged circulation, and was, thus, selected for evaluation in arthritic mice.

Enhanced passive accumulation of the 54 kDa dye-grafted p(HPMA-co-AzMA) in the joints of arthritic mice compared to healthy was demonstrated (Figure 3 and Figure 4) with the bioavailability in arthritic tissue (AUC = 1783% · h) being ~12 times higher ($P = 0.01$) compared to healthy tissue (AUC = 148% · h) (see Additional file 2: Figure S2). Additionally, at 24 h, polymer levels in the blood of arthritic animals were lower than that of healthy (see Additional file 3: Figure S3), but showed a higher level in arthritic paws compared to healthy (Figure 3 and Figure 4). This suggests the polymer to be escaping blood circulation faster in the arthritic mice, probably due to accumulation in the arthritic joints. The polymer levels in healthy mice joints initially increased by 63% within 10 minutes but subsequently quickly declined, probably as a result of blood clearance of the polymer in general and a healthy microvasculature not exhibiting permeability and polymer extravasation effects. Free dye in arthritic or non-arthritic tissue was rapidly excreted and almost cleared before the first time point at 10 min (Figure 3, 10 min).

The arthritic scores, as an indicator of inflamed severity, were not entirely uniform with individual mice exhibiting combinations of joint scores (1–4). A Spearman test of polymer accumulation (at 24 h) and arthritic score correlation was, therefore, performed (Figure 5), supporting strong correlation between polymer accumulation and the arthritic condition ($r = 0.89$, $P < 0.0001$).

In summary, this work demonstrates the *in vivo* characteristics of a new azide containing copolymer, (p(HPMA-co-AzMA)), which offers a more versatile and well-defined alternative to existing bioconjugate systems. Attractive

in vivo properties such as prolonged blood retention and specific accumulation in inflamed joints of RA illustrate the potential of the material for systemic delivery of anti-inflammatory drugs for local effects in the treatment of RA.

Additional files

Additional file 1: Figure S1. Blood plasma bioavailability (AUC), in healthy BALB/c mice, of p(HPMA-co-AzMA) of 15, 36 and 54 kDa calculated from blood plasma polymer profiles in Figure 2.

Additional file 2: Figure S2. Bioavailability (AUC) of dye-labeled p(HPMA-co-AzMA) of 54 kDa and free dye in arthritic and healthy joints. AUC's are calculated from p(HPMA-co-AzMA) joint accumulation profiles in Figure 3.

Additional file 3: Figure S3. Blood plasma levels of dye-labeled p(HPMA-co-AzMA) and free dye at 24 h. Polymer levels in the blood indicate that the polymer escapes blood circulation faster in the arthritic mice possible due to accumulation in the arthritic mice joints. Levels are normalized to the highest mean value and averages shown with SEM.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MFE and KAH provided conception of research and drafted the manuscript; MFE, KAH, KB and BWD, planned the study; MFE and KB performed experiments; MFE and BWD analyzed and interpreted data; MFE prepared figures. All authors read and approved the final manuscript.

Acknowledgements

We thank the Lundbeck Foundation for supporting this work through the grant: Lundbeck Foundation Nanomedicine Center for Individualized Management of Tissue Damage and Regeneration.

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Received: 24 June 2014 Accepted: 3 September 2014

Published: 11 September 2014

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doi:10.1186/2052-8426-2-29

Cite this article as: Ebbesen *et al.*: Extended blood circulation and joint accumulation of a p(HPMA-co-AzMA)-based nanoconjugate in a murine model of rheumatoid arthritis. *Molecular and Cellular Therapies* 2014 **2**:29.

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