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Gold nanoparticle–antibody conjugates for specific extraction and subsequent analysis by liquid chromatography–tandem mass spectrometry of malondialdehyde-modified low density lipoprotein as biomarker for cardiovascular risk

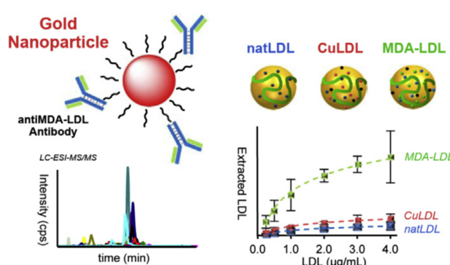
Elisabeth Haller [a], Wolfgang Lindner [a], Michael Lämmerhofer [b,*]

[a] Department of Analytical Chemistry, University of Vienna, Währingerstrasse 38, 1090 Vienna, Austria
[b] Institute of Pharmaceutical Sciences, Pharmaceutical (Bio)Analysis, University of Tübingen, Auf der Morgenstelle 8, 72076 Tübingen, Germany

HIGHLIGHTS

- Generic platform for nanoimmunoaffinity extraction of biomarkers developed.
- Application to extract malondialdehyde-modified low-density lipoprotein.
- After extraction (oxidized) (phospho) lipids are analyzed by LC–MS/MS.
- Comparison of random and oriented immobilization of antibodies on gold nanoparticle.
- Optimized binding properties for oriented immobilization via Cys-modified protein A.

GRAPHICAL ABSTRACT



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ABSTRACT

Oxidized low-density lipoproteins (OxLDLs) like malondialdehyde-modified low-density lipoprotein (MDA-LDL) play a major role in atherosclerosis and have been proposed as useful biomarkers for oxidative stress. In this study, gold-nanoparticles (GNPs) were functionalized via distinct chemistries with anti-MDA-LDL antibodies (Abs) for selective recognition and capture of MDA-LDL from biological matrices. The study focused on optimization of binding affinities and saturation capacities of the antiMDA-LDL-Ab-GNP bioconjugate by exploring distinct random and oriented immobilization approaches, such as (i) direct adsorptive attachment of Abs on the GNP surface, (ii) covalent bonding by amide coupling of Abs to carboxy-terminated-pegylated GNPs, (iii) oriented immobilization via oxidized carbohydrate moiety of the Ab on hydrazide-derivatized GNPs and (iv) cysteine-tagged protein A (cProtA)-bonded GNPs. Depending on immobilization chemistry, up to 3 antibodies per GNP could be immobilized as determined by ELISA. The highest binding capacity was achieved with the GNP-cProtA-Ab bioconjugate which yielded a saturation capacity of $2.24 \pm 0.04\ \mu g\ mL^{-1}$ GNP suspension for MDA-LDL with an affinity K_d of $5.25 \pm 0.11 \times 10^{-10}$ M. The GNP-cProtA-antiMDA-LDL bioconjugate revealed high specificity for MDA-LDL over copper(II)-oxidized LDL as well as native human LDL. This clearly demonstrates the usefulness of the new GNP-Ab bioconjugates for specific extraction of MDA-LDL from plasma samples as biomarkers of oxidative stress.

Abbreviations: LDL, low-density-lipoproteins; OxLDL, oxidized low-density-lipoproteins; ROS, reactive oxygen species; PUFAs, polyunsaturated fatty acids; MDA, malondialdehyde; 4-HNE, 4-hydroxynonenal; POVPC, 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine; ALEs, advanced lipid peroxidation end products; apo B-100, apolipoprotein B-100; OSE, oxidation-specific epitopes; GNPs, gold nanoparticles; cProtA, cysteine modified protein A.

* Corresponding author. Tel.: +49 7071 29 78 793; fax: +49 7071 29 4565.
E-mail address: Michael.laemmerhofer@uni-tuebingen.de (M. Lämmerhofer).

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Their combination as specific immunoextraction nanomaterials with analysis by LC–MS/MS allows sensitive and selective detection of MDA-LDL in complex samples.

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1. Introduction

Early states of inflammatory diseases are characterized by evaluated levels of oxidized low-density-lipoproteins (OxLDL) [1–7]. Different forms of OxLDL have major impact in the initiation and progress of atherosclerosis which is a systemic chronic inflammatory disease and one of the major causes of morbidity and mortality in the western world. It is therefore of particular interest for clinical diagnosis for epidemiological reasons [8]. Under conditions of oxidative stress [9], excess of reactive oxygen species (ROS) leads to oxidative damage of biomolecules such as lipids containing polyunsaturated fatty acids (PUFAs) [10]. As a result, a plethora of lipid peroxidation products are formed, including malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), lipid hydroperoxides (*e.g.*, 13-hydroxyperoxyoctadecadienoic acid) and oxidized phospholipids like 1-palmitoyl-2-(5-oxovaleroyl)-*sn*-glycero-3-phosphocholine (POVPC) to mention a few. Further, generated reactive aldehydes can react with cysteine, histidine, and lysine residues of proteins, forming advanced lipid peroxidation end products (ALEs) through Schiff bases or Michael type addition [11,12]. For example, MDA can react with the ε-amino group of lysine residues of proteins such as apolipoprotein B-100 (apo B-100) and such a reaction product was found in atherosclerotic lesions [13–22]. All these oxidative modifications represent oxidation-specific epitopes (OSEs) and are immunogenic, pro-inflammatory and pro-atherogenic [10]. In fact, atherosclerosis involves subendothelial accumulation of LDL (especially if elevated levels are circulating in the blood stream) and its oxidation by ROS. Oxidized forms of LDL are recognized by scavenger receptors of macrophages such as SR-A, CD36 and CD68 [8,11,23,24]. The enhanced uptake of OxLDL by macrophages leads to the generation of lipid-laden macrophage-derived foam cells and ultimately advanced atherosclerotic lesions, plaques and thrombosis [8,25–28].

Thus, monitoring of oxidative damages of biomolecules may be essential for profound understanding of atherosclerosis and other age related diseases [8,29]. Since MDA-LDL is a commonly accepted cardiovascular risk factor, it could have great potential as biomarker of systemic oxidative stress and prognostic risk assessment of atherosclerosis [6,30–33]. In fact, recent literature emphasizes that circulating MDA-LDL may be a more useful clinical biomarker for vascular inflammation within atherosclerotic plaques than for instance high-sensitivity C-reactive protein (hsCRP) or asymmetric dimethylarginine [34]. ELISA-based bioassays have already been developed for the determination of OxLDL and MDA-LDL, respectively, and are routinely used in clinical research [2,15,35–37]. Other methods are rarely used to analyze MDA-LDL and comprise GCMS for analysis of MDA-lysine in MDA-LDL [38], thiobarbituric acid assay for estimation of the degree of MDA-modification in MDA-LDL [38], mapping of modifications on ApoB-100 by analysis of tryptic digests and use of narrow mass-window high-resolution MS hyphenated to nanoHPLC [39]. In general, ELISA assays are usually used for MDA-LDL analysis. However, ELISA assays suffer amongst others from false positive results. Furthermore, such immunoassays give just a quantitative signal but do not allow to derive information on the pattern of oxidized species present in OxLDL and MDA-LDL particles, respectively. However, it is well-known that individual oxidized lipid species such as oxidized phospholipids (OxPLs) with various oxidative modifications of PUFAs are not equivalent in their bioactivities and may generate significantly distinct responses in various signaling pathways [40,41]. Thus, a more differentiated picture could be gained by bioassays which allow distinction and quantification of individual oxidized lipids, in particular OxPLs. To account for this, we herein propose a concept for analysis of OxPL biomarkers of oxidative stress by nano-immunoaffinity extraction of oxidized-LDL and subsequent analysis of the (oxidized) lipid fraction by LC–MS/MS. MDA-LDL serves herein as model antigen for oxidized LDL particles. The employed standard of MDA-LDL is obtained by coupling of malondialdehyde to the ε-lysine group of apoB-100. Thus, no lipid changes should be present in the employed MDA-LDL particles and it is therefore not present as such in plasma. Plasma MDA-LDL results from lipid oxidation which generates truncated PLs and MDA that in turn can modify apoB-100 (and other proteins). It is hence sometimes criticized that MDA-modified LDL is not suitable clinically as it represents just a model particle that does not exist in plasma. Abs raised against this antigen would show only little cross-reactivity towards other oxidized LDL particles limiting their usefulness. Moreover, MDA-LDL is a high variability antigen with limited reproducibility [42]. Yet the antigenicity of anti-MDA-LDL Abs stems from MDA-type epitopes and hence it is supposed that anti-MDA-LDL Abs used in this study can capture also plasma MDA-LDL and not only the employed MDA-modified LDL standard used to develop this assay. In fact, this hypothesis is supported by a recent study which showed that MDA-LDL is not required to generate immunogens but oxidation-specific epitopes on OxLDL are sufficient [43]. In any case, it was suitable to develop a general Ab-GNP based immunoaffinity nanotrapping platform which might be easily transferred to capture other oxidized LDL particles using other Abs (*e.g.*, anti-copper-oxidized LDL Abs).

Along this line, the prime goal of this study was dealing with the tuning of the chemistry for immobilization of Abs in particular anti-MDA-LDL antibody, on colloidal gold nanoparticles (GNPs) in order to develop an efficient generic immunoaffinity nanotrapping approach and optimize affinities, maximize binding capacities, reduce non-specific interactions, improve specificity for MDA-LDL and reduce cross-reactivity. The workflow is straightforward and involves simple pipetting and spinning steps for isolation of the antigen. The pellet of the GNP-Ab conjugate with bound antigen is subsequently extracted with methanol. GNPs and proteins precipitate. The methanolic extracts containing the lipids can be conveniently analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS). This analysis concept has great potential for clinical diagnostics of oxidative stress. GNP-Ab conjugates assure selective extraction of the desired antigen and subsequent analysis with mass spectrometry enables highly sensitive detection of the entire pattern of oxidized lipids and precludes false positive results of ELISA assays due to the additional level of specificity by selective mass spectrometric scanning modes or filtering techniques. While immunoaffinity based analysis concepts are widely used in environmental and diagnostic immunoassays [44–47], biochemical studies, for immunosensors [48], as tool for specific microdialysis [49], in nanomedicine and biomedical applications [44,50,51], the utilization of GNP-Ab conjugates as sample preparation strategy for MS analysis has been less frequently reported [52,53]. In general, however, MS-assisted immunological assays were first described by the Hunt laboratory in 1991 and were recently reviewed for proteomics by Madian et al. [54]. Since the chemistry of resultant bioconjugates is decisive for the functionality of GNP-Ab bioconjugates for the particular purpose and a plethora of distinct strategies differing in

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